DETECTION OF P16^{INK4a} IN OROPHARYNGEAL AND UPPER RESPIRATORY TRACT SQUAMOUS CELL CARCINOMA

Dissertation submitted in
Partial fulfillment of the requirements for the award of

M.D. DEGREE

in

PATHOLOGY – BRANCH III

THE TAMILNADU
DR. M.G.R. MEDICAL UNIVERSITY
CHENNAI

APRIL 2016

DECLARATION

I hereby declare that the dissertation entitled "**Detection of p16^{INK4a} in Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinoma**" was done by me in the Department of Pathology, Chengalpattu Medical College from June 2014 to August 2015 under the guidance and supervision of **Dr. S. Ravi, M.D**., Professor and Head, Department of Pathology, Chengalpattu Medical College.

This dissertation is submitted to the Tamil Nadu Dr.MGR Medical University, Chennai towards the partial fulfillment of the requirements for the award of M.D.Degree in Pathology.

I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

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This is to certify that the dissertation entitled "**Detection of $p16^{INK4a}$ in Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinoma**" is a record of bonafide work done by **Dr.M. Johnraj Suresh** in the Department of Pathology, Chengalpattu Medical College, Chengalpattu under the supervision of **Dr. S. Ravi M.D.,** Professor and Head , Department of Pathology and submitted in partial fulfillment of the requirements for the award of M.D. Degree in Pathology by The Tamilnadu Dr. MGR Medical University, Chennai. This work has not previously formed the basis for the award of a degree or diploma.

Dr.K. Muthuraj M.S.
Dean,
Chengalpattu Medical College,
Chengalpattu.

Dr. S. Ravi M.D.
Professor and Head,
Department of Pathology,
Chengalpattu Medical college,
Chengalpattu.

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Dr. S. Ravi M.D.
Professor and Head,
Department of Pathology,
Chengalpattu Medical College,
Chengalpattu.

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Dear Dr.

The Institutional Ethical Committee of Chengalpattu Medical College reviewed and discussed your application to conduct the clinical / dissertation work entitled

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2. Patient information sheet and informed consent form in English and / or
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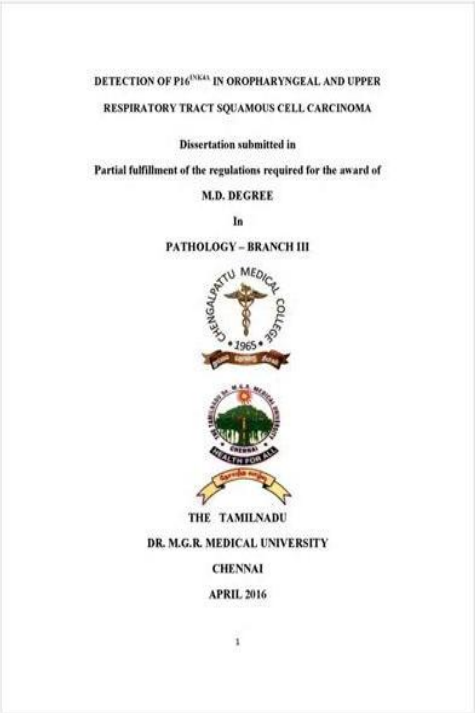

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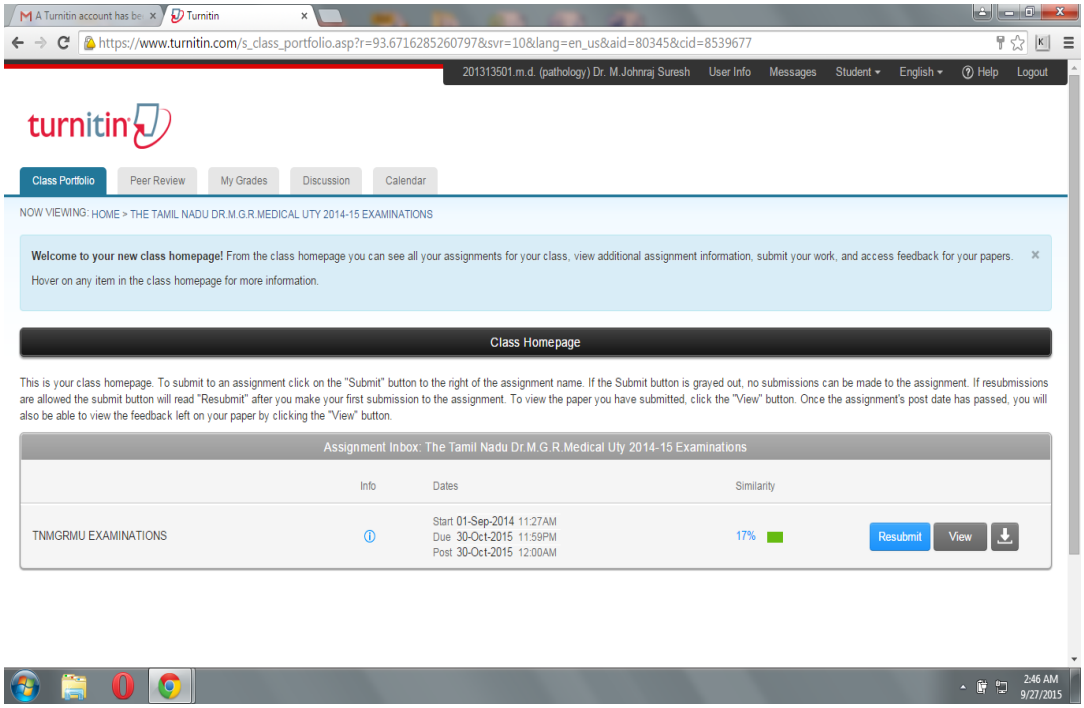
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ABSTRACT

BACKGROUND

The incidence of HPV associated HNSCC is increasing over the past 30 years. It is a growing public health concern. It has been reported that tissues of HPV associated HNSCCs over express p16INK4a . Therefore p16INK4a is used as a surrogate marker to detect HPV associated HNSCC. Immunohistochemical detection of p16INK4a is an easy and simple technique than molecular detection of HPVs. Hence we investigated the presence of p16INK4a in HNSCCs.

OBJECTIVES

The objectives of our study are (1)To study the association of p16INK4a expression with HNSCC, thus with the HPV. (2)To compare the p16INK4a expression in different subsites of the HNSCC. (3)To correlate the level of p16INK4a expression with different grades of HNSCC.

MATERIALS AND METHODS

A total sample of 60 cases were analysed during the period of June 2014 to August 2015. We performed IHC detection in sections of formalin fixed paraffin embedded tissue of HNSCC cases and correlated the various patterns of p16INK4a positivity with respect to histopathological diagnosis.

RESULTS

In the present study, 75% of the HNSCC cases were above 50 years of age. HNSCC was more common in males with male to female ratio of 6:1 .

93.33% of HNSCC cases were positive for p16INK4a, of which the most common pattern was diffuse nuclear and cytoplasmic staining (53.33%).

CONCLUSION

In the present study, increased number of HNSCC cases were seen over expressing p16INK4a (93.33%). Oropharynx was the most common site for p16INK4a positivity in HNSCC cases (94.44%). Among the oral cavity SCC cases, tongue was the most common site involved (64%). Of the HNSCC cases, most cases (53.33%) had diffuse pattern of p16INK4a over expression. However, DNA detection based studies are needed to validate the utility of IHC detection of p16INK4a as a surrogate marker for HPV associated HNSCC.

Key words

HNSCC, p16INK4a, HPV, Head and neck cancer, Squamous cell carcinoma

INTRODUCTION

Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinoma or Head and Neck Squamous Cell Carcinoma (HNSCC) is a global health problem with high prevalence and mortality rate across the world. Every year approximately 6,00,000 cases have been reported in HNSCC, worldwide[1]. This is the sixth most common cancer group, causing 2,00,000 deaths every year. The incidence is much higher in India, Southeast Asia and Europe[2]. It is the top ranked among all cancers in males and got third in females[3]. Almost 90% of carcinoma reported in HNSCC. It usually develops from oropharynx, oral cavity, larynx and hypopharynx. Oral lesions like leukoplakia, erythroplakia, and lichen planus have an increased risk for transformation to malignancy in oral cavity[4]. The distribution of oral carcinoma in India varies from state to state. The prevalence of oral carcinoma across the country has shown marked increase in Uttar Pradesh, Madhya Pradesh, Gujarat, Bihar and Maharashtra[5].

The risk factors of HNSCC are multifactorial; many epidemiological study data commend the association between the HNSCC with tobacco use, active and passive. Smokeless tobacco usage and alcohol consumption are also important causes[6,7]. In the last two decades the study about Human Papilloma Virus (HPV) in HNSCC showed a unique cause of malignancy[8]. Uterine cervix carcinogenesis risk factor HPV is now well recognized as the risk factor of HNSCC, which shown a marked increased prevalence rate[9]. The transmission of HPV infection through body fluids, oral sex is an important

transmission type. The concurrence of HPV in HNSCC was established by Syrjanen et al. in 1983 and many researchers supported the fact on the basis of evidence.

Statistical data collected about the prevalence of HPV in HNSCC in 2013 shown 36% of contribution and it is a double the value of previous decade data[10]. It is estimated, after 5 years HNSCC will overcome the prevalence of Cervical Carcinoma. It is more prevalent in younger people, less than 60 years in whom there is a decreasing rate of smoking and increase in the transmission of HPV among youngsters. The increased incidence rate shown in the age of 40 to 50 years without any environmental risk factors, is associated with the continual infection with HPV[11,12]. Many researchers were working on determining the possible viral causing factor, mainly the oncogenic HPV. The effect of high alcohol consumption which may also leads to the HPV infection due to the alteration of mucosal membrane[13].

More than 99% of cervical carcinoma is due to the HPV 16, 18 and 33 subtypes, globally. The epithelial cell specific HPV is a group of host specific DNA virus with more than 120 of subtypes, almost 4 subtypes has been studied in cervical carcinoma. Subtypes 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89 are considered as low oncogenic effect viruses which can be isolated from low grade epithelial cell[14,15,16] . HPV 16 is the commonest subtype in HNSCC. There is an increased risk of HNSCC in the patient diagnosed with cervical carcinoma. The HPV infection is also traced in benign oral lesions as condyloma acuminata, common warts, oral papilloma, focal epithelial hyperplasia and in potentially malignant or premalignant

erythroplakia, erythro-leukoplakia and leukoplakia, and proliferative verrucous leukoplakia[17]. The exact origin of HPV infection in HNSCC is not clear; it usually starts from the reticulated epithelium of oropharynx, which lines the crypts of the palatine tonsils and base of tongue. The irregular and deep shaped crypts in the tonsils allow for the exposure of HPV pathogen within the lymphoepithelial tissue[18,19] .

The mechanism of HNSCC in HPV negative patients is frequent DNA mutation. HPV positive HNSCC are due to the genetic alterations. The oncogenic proteins E6 and E7 released by the high risk virus subtypes interrupt the p53 and pRb tumor suppressing pathways respectively, which leads to increased cell proliferation and genomic instability leading to carcinogenesis. $p16^{INK4a}$ is one of the several cyclin-dependent kinase inhibitors responsible for regulation of normal cell cycle. As pRb is inactivated by E7 protein, cells are released from growth-suppressive stimuli mediated by the $p16^{INK4a}$. Thus reduced or lost pRb function results in enhanced $p16^{INK4a}$ levels, as a result of a negative feedback control[9]. $p16^{INK4a}$ is commonly used as a biomarker for transcriptionally active HPV-associated cancers[20,21,22].

AIMS AND OBJECTIVES

1. To study the association of $p16^{INK4a}$ expression with head and neck squamous cell carcinoma, thus with the Human papilloma virus.

2. To compare the $p16^{INK4a}$ expression in different sites of the head and neck squamous cell carcinoma.

3. To correlate the level of $p16^{INK4a}$ expression with different grades of head and neck squamous cell carcinoma.

REVIEW OF LITERATURE

Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinoma or Head and Neck Squamous Cell Carcinoma (HNSCC) is the world fifth most common cancer in worldwide. Squamous Cell Carcinoma is the most common malignancy which has high incidence of more than 600000 cases every year and high morbidity worldwide[1,2] . Squamous cell carcinoma occurs in 5 anatomical sites oral cavity, oropharynx, nasopharynx, hypopharynx and larynx[4] . Human papilloma virus (HPV) positive HNSCC is highly associated with oral sex behavior. The main risk factors of squamous cell carcinomas are tobacco users, alcohol users and oral sex[23] .

There is an association between HPV positive HNSCC with sexual behavior, but not in HPV negative HNSCC [24] . Tobacco use is the main cause of HNSCC with as high as 80% of cases attributed to it. Alcohol usage acts synergistically with tobacco in the increased incidence of HNSCC[25]. There is a decrease in the incidence of HNSCC due to reduction of tobacco use but there is a remarkable increase in the incidence due to HPV infection[26] . HPV infected HNSCC has genetic alteration, influenced by HPV oncoproteins E6 and E7 which inactivate the tumor suppressing gene[27] .

Historical aspects

Papilloma viruses were first identified from rabbits in 1933. They were transmissible and found to cause benign papillomas.

HPV was first identified in 1956 and associated with a variety of benign growths in humans[28] .

HPV has subsequently been shown to be the cause of many types of human cancer.

HPV was first implicated in cancer biology forty years ago by Harald zur Hausen. He was awarded Nobel Prize in Physiology or Medicine in 2008 for the discovery of HPV causing cervical cancer. After that, HPV is also associated with other anogenital cancers like vulvar, penile and anal, mainly due to sexual transmission. Recently, high risk HPV is detected in HNSCCs, particularly in the oropharynx, previously caused mainly by tobacco and alcohol consumption[29] .

Gissmann et al first described the HPV presence in the head and neck region in 1982. They noted HPV DNA in patients with laryngeal papillomas. Then, Syrjanen et al detected HPV antigens in oral squamous cell lesions in 1983.

In 1989, Brandsma and Abramson detected HPV 16 DNA in the oropharynx, specifically the palatine tonsils, in two of the seven tonsillar squamous cell carcinomas[30] .

Since then more data have come, implicating HPVs in the oncogenesis of HNSCC, predominantly OPSCC.

Normal anatomy[31]

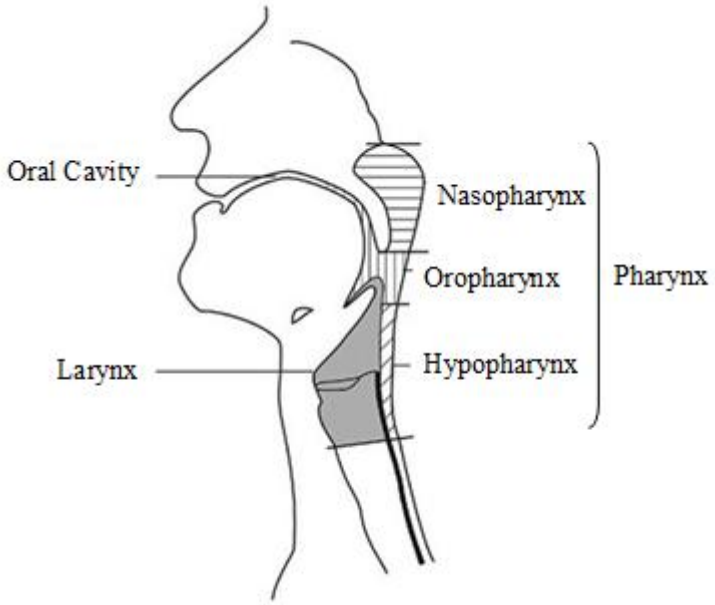

Fig 1. Head and neck region

Oral cavity and posterior tongue

Most of the oral cavity is lined by non keratinized, stratified squamous epithelium except for certain regions where the epithelium becomes keratinized.

Non Keratinized mucosa constitutes from the basement membrane to the surface, stratum basale stratum spinosum (prickly) and a superficial layer.

In Keratinized epithelium, stratum granulosum and stratum corneum (keratinized layer) are existing above the stratum spinosum.

Molecular study show stratum basale are the only cell type which expresses proliferation allied antigens and the RNA component of telomerase.

These basal cells replace the dedicated cells that undergo terminal differentiation in the major superficial layers of the epithelium. Thus basal cells ensure the epithelium's turnover and have the role of stem cells.

Regional variations in the composition of the epithelium like its quantity of keratinization reflect differences in the amount of mechanical stress during mastication which depends on the resiliency of the exposed areas.

Buccal mucosa has extended mucosal ridges anchoring it to severly collagenized lamina propria. Areas secured from stress such as the floor of mouth, possess thinner and shallower rete ridges and less collagenized lamina propria.

In the posterior third of tongue lingual mucosa has lymphoid tissue, part of the mucosa – associated lymphoid tissue.

The level of keratinization, thickness, presence of pigments and the extent of vascularization of mucosa and lamina propria concern the colour of the mucosa. These features are effective when correlating the clinical appearance of mucosal lesions with their microscopic appearance.

Oral cavity has small salivary glands within its submucosa. Intra epithelial neoplasia together with in situ carcinoma, can involve both the acini and excretory ducts of minor salivary glands which can mimic invasive carcinoma.

Lip

Lymphatics of the central part of the lower lip drain to the sub mental nodes. Lymphatics from the rest of the lower lip pass to the submandibular nodes.

Cheek

Lymphatics of the cheek drain chiefly into the submandibular and preauricular nodes and party also to the buccal and mandibular nodes.

Floor of the mouth

Lymphatics from the anterior part of the floor of the mouth pass to the submental nodes. Those from the hard palate and soft palate pass to the retropharyngeal and upper deep cervical nodes. The gums and the rest of the floor drain into the submandibular nodes.

Gums

Lymphatics of the upper gums pass to the submandibular nodes. The anterior part of the lower gums drains into the submental nodes, whereas the posterior part drains into the submandibular nodes.

Hard palate

The blood supply of the hard palate is from the greater palatine branch of maxillary artery.

The venous drainage is into the pterygoid plexus of veins.

The lymphatics of the hard palate drain mostly to the upper deep cervical nodes and partly to the retropharyngeal nodes.

Soft palate

Blood supply of the soft palate is from the Greater palatine branch of maxillary artery, Ascending palatine branch of facial artery and Palatine branch of ascending pharyngeal artery.

Venous drainage pass to the pterygoid and tonsillar plexuses of veins. Lymphatic of the soft palate drain into the upper deep cervical and retropharyngeal lymph nodes.

Tongue

Arterial supply is by the lingual artery a branch of the external carotid artery. Root of the tongue is also supplied by the tonsillar branch of facial artery and ascending pharyngeal branch of external carotid artery.

Venous drainage of the tongue: Two venae comitantes accompany the lingual artery and one vena comitantes accompanies the hypoglossal nerve.

Deep lingual vein is the largest and principal vein of the tongue. These veins unite at the posterior border of the hyoglossus to form the lingual vein which ends in the internal jugular vein.

Lymphatic drainage of the tongue: Tip of the tongue drains bilaterally to the submental nodes.

Right and left halves of the remaining part of the anterior two-thirds of the tongue drain unilaterally to the submandibular nodes. A few central lymphatics drain bilaterally to the same nodes.

Posterior one third of the tongue drains bilaterally to the jugulo-omohyoid nodes, these are known as lymph nodes of the tongue.

Posterior most part of the tongue drains bilaterally into the upper deep cervical lymph nodes.

Pharynx

Pharynx is divided into three compartments: Oropharynx, nasopharynx and hypopharynx.

Both oropharynx and hypopharynx are lined by stratified non keratinized squamous epithelium with submucosal compartments having seromucinous glands and aggregates of lymphoid tissue.

In the nasopharynx 60% is lined by stratified non keratinized squamous epithelium and leftover 40% by pseudostratified, ciliated, respiratory type epithelium.

The pseudostratified, ciliated, respiratory type epithelium predominates in the posterior nares and in the roof of posterior wall.

Other areas reveal an alteration of the two types of epithelia.

At the alteration between the two types, the mucosa has an intermitted or transitional appearance that may mimic intraepithelial neoplasia.

Low magnification of the mucosa in these areas may appear a moderately disorganized architecture, also called as incomplete metaplasia, which may mimic true dysplasia.

Higher exaggeration classically reveals preserved maturation and lack of frank nuclear atypia, often coupled with the presence of ciliated layer in the vicinity of these areas.

Similar characters are seen at the transition between squamous and respiratory epithelia in the larynx in regular conditions and in squamous metaplasia in the bronchial ciliated epithelium in response to irritants.

Palatine Tonsil

Arterial supply of the tonsil is mainly from Tonsillar branch of facial artery. Additional sources of arterial supply are

Ascending palatine branch of facial artery, Dorsal lingual branches of the lingual artery, Ascending pharyngeal branch of the external carotid artery and Greater palatine branch of the maxillary artery.

Venous drainage pass to the palatine, pharyngeal or facial veins.

Lymphatic drainage pass to jugulodigastric node.

Pharynx

Arterial supply of the pharynx are Ascending pharyngeal branch of the external carotid artery, Ascending palatine and tonsillar branches of the facial

artery, Dorsal lingual branches of the lingual artery and Greater palatine, Pharyngeal and pterygoid branches of the maxillary artery.

Venis form a plexus on the posterolateral aspect of the pharynx. The plexus receives blood from the pharynx, soft palate and the prevertebral region. It drains into the internal jugular and facial veins.

Lymphatics of the pharynx drains into the retropharyngeal and deep cervical lymph nodes.

Larynx

Normal laryngeal mucosal lining also varies to some extent in thickness, like in the oral cavity. Thick keratinizing surface epithelial lining is controlled to laryngeal glottis. The thicker keratinized epithelium of the glottis prevents the mucosa from repetitive mechanical trauma of phonation.

The remaining epithelial lining of the larynx changes according to the location and shows alteration of ciliated, respiratory type and squamous epithelia.

The supraglottic compartment extends from the tip of the epiglottis to the inferior border of false vocal cord. It shows respiratory epithelium.

True vocal cords are lined by squamous epithelia.

The subglottic larynx is the portion of larynx among the lower border of true vocal cord and first tracheal ring. It is lined by the respiratory mucosa.

The respiratory epithelium is ciliated pseudostratified epithelium. Similar to squamous mucosa, the basal cells are the regenerative part of the respiratory epithelium. The modified cells in the luminal surface are composed of ciliated, brush and goblet cells, allowing mucociliary clearance. A minor component of the epithelium is constituted by small granular cells, have neurosecretory granules and belong to the diffuse neuroendocrine system. These cells are detected only by electron microscopy or special immuno histo chemical stains.

Like in the pharynx, areas between the squamous and respiratory- type epithelia have transitional appearance.

In nearly half of smokers, patches of squamous metaplasia are seen in the supraglottic larynx.

Arterial supply up to the vocal folds is by the superior laryngeal artery, a branch of the superior thyroid artery.

Below the vocal folds is supplied by the inferior laryngeal artery, a branch of the inferior thyroid artery.

Venous drainage up to the vocal folds is by the superior laryngeal vein which drains into the superior thyroid vein.

Below the vocal folds is by the inferior laryngeal vein which drains into the inferior thyroid vein.

Lymphatics from above the vocal folds drain along the superior thyroid vessels to the anterosuperior group of deep cervical nodes.

Below the vocal folds drain to the postero inferior group of deep cervical nodes. A few of them drain through the prelaryngeal nodes.

Epidemiology

The association of HPV infection in HNSCC has attracted the researchers in the past 25 years due to the high incidence rate. The incidence of HNSCC in many countries has been increased significantly. In 1970 the incidence of HPV infected tonsillar and carcinoma in the base of the tongue has been reported by Nasman et al. in the Stockholm region. The incidence rate has been increased from 23% in 1970 to 93% in 2007[32] . The concomitant increase of HPV infected HNSCC than tobacco associated HNSCC is due to the reduction on tobacco usage among youngsters group. The increase in the HPV associated HNSCC is due to the sexual behavior changes among the youngsters. Oral sex is most common among youngsters group which relate to the incidence of HPV infection[33] . HPV infected Oropharynx carcinoma incidence in higher than the other anatomical site, it is reported that oropharynx is five times more likely to be infected by HPV than oral cavity, larynx[34] . The prevalence of HPV infection in different population group has been studied - 9.3% in Sweden, 2.3% in Australia, 0.6% in Japan, 6.95% in United States[35] . All HPV infections won't progress to carcinoma. Slow clearance of virus has been shown to be risk on the development of anogenital carcinoma. HPV infected Oropharyngeal Squamous Cell Carcinoma (OPSCC) has been increasing significantly. In the 2008 report, among 85000 of OPSCC, 22000 cases are reported as HPV positive. There is a increase of 225% HPV infected HNSCC cases from 1988 to 2004 in United states with 50% decreases

in HPV negative cases[36] . The same trend has been reported in Europe and Australia. The overall incidence rate of HPV positive OPSCC is 25.6% worldwide, which may vary according to geographical region. The proportion of HPV-positive OPSCC was 56% in North America; 52% in Japan; 45% in Australia; 39% in Western and Northern Europe; 38% in Eastern Europe; 17% in Southern Europe; and 13% in the rest of the world[35]. The incidence of HPV positive HNSCC is higher among persons having multiple sexual partners, middle aged, not using tobacco or alcohol. HPV positive cases usually clear in own but some of the viral DNA integrates with host genome; this is the main cause of inducing HPV carcinogenesis. The survival rate is high in HPV positive HNSCC cases than HPV negative cases[37] .

Squamous cell carcinoma of the Oral cavity

Carcinomas of oral cavity are commonly situated in the base of the tongue and tonsils. These tumours are mostly undifferentiated and solid and resemble large cell malignant lymphoma. The epithelium surrounding the tumour shows dysplastic changes. The squamous cell carcinomas that show eosinophilic infiltration are associated with good prognosis. Perineurial and vascular invasions are common if immunohistochemical markers are done[38].

Squamous cell carcinoma of the Oropharynx

The squamous cell carcinoma of oropharynx are less differentiated when compared to squamous cell carcinoma of oral cavity.

Squamous cell carcinoma of the Larynx

Laryngeal carcinomas usually occur as a protruding mass of pink or gray colour and they are usually ulcerated. Lesions of the vocal cord are keratotic. Nearly 90% of the laryngeal carcinomas are squamous cell type. On the degree of differentiation, cellular pleomorphism and mitotic activity they are classified into well, moderately and poorly differentiated[39]. The tumour that are situated in the subglottic region are smaller in size are well differentiated. The tumour that has minimal stromal invasion, within 0.5mm from the basement membrane are called as minimally invasive, microinvasive or superficially invasive. The mobility of cord is not affected. The evolution of invasive carcinoma from in situ carcinoma is associated with replacement of CD-34 positive stromal cells by smooth muscle positive myofibroblasts.

Variants of Squamous cell carcinoma

I. Verrucous Carcinoma

In oral cavity verrucous carcinoma are marcroscopically, seen as a large fungating, soft papillary growth. They are often infected and invade the soft structure of the cheek and penetrate the maxilla or mandible and invade the perineurial tissues. They invade the nodes very rarely.

Verrucous carcinoma are well differentiated and so it is difficult to diagnose it microscopically. A superficial biopsy will show benign papillomatosis. Adequate biopsy will show swollen and voluminous rete pegs extending into the deeper tissues and have complex pattern[40]. The good differentiation through out the tumour differentiate if from squamous cell

tumour. The cells of verrucous carcinoma are larger than the cells of squamous cell carcinoma. In 20% of the cases foci of squamous cell carcinoma occurs with in verrucous carcinoma. This type usually have a high rate of recurrence.

In the larynx also they are macroscopically a polypoidal growth and microscopically they are well differentiated. They invade locally but rarely have distant metatasis[41]. Verrucous carcinoma closely resembles verrucous hyperplasia. They are differentiated by the presence or absence of invasion and it is difficult to be diagnosed with a small blopsy. Hybrid carcinoma also occur in laryngeal cancer. Surgery is the means of treatment. Radiation therapy was given when there is an anaplastic transformation.

II. Adenoid (Pseudoglandular) squamous cell carcinoma

Mostly they are situated in the lip and the underlying factor is actinic radiation. They may rarely present in gingiva or tongue where actinic radiation have no role[42]. They have a pseudoglandular or alveolar appearance.

III. Adenosquamous carcinoma

These are rare tumours. They have squamous differentiation amidst true glandular differentiation[43,44]. Some tumours may be from minor salivary glands but they are different from mucoepidermoid carcinomas.

IV. Basaloid squamous cell carcinoma

In oral cavity, this is the most aggressive from. They commonly occur in the oral cavity, oropharynx, oesophagus and larynx. They may occur rarely

in the lung also. The ‘cloacogenic carcinoma’ of the anal canal may also resemble basaloid carcinoma. They mildly resemble adenocarcinoma. Histogentically they mimic adenosquamous carcinoma but microscopically they are different. Basaloid carcinoma have areas of clear squamous differentiation mixed with solid tumour and have peripheral palisading and a thick basement membrane[45].

They have cystic spaces filled with mucoid and hyaline material and they resemble adenoid cystic carcinoma. The classical feature of this tumour is the prominence of basal lamina at both ultrastructurally and immunohisto chemically. The tumour immunoreact for high molecular weight keratin (detected with the 34βE12 antibody) consistently.

In the larynx also it has an aggressive course. It usually occurs in heavy smokers. It is characterized by hyperchromatic nuclei, scanty cytoplasm, necrosis and peripheral palisading. There is an attempt to differentiate torwards glandular structures. This tumour may also occur in tongue, pharynx and oesophagus. It should not be confused with adenoid cystic carcinoma.

V. Papillary Squamous Cell Carcinoma

It is usually found in the oropharynx of elderly patients and it is usually HPV positive.

In the larynx it occurs as an exophytic growth. It is usually associated with human papilloma virus and have a good prognosis when compared to the one in sinonasal region[46] . It is differentiated from verrucous carcinoma by its cytological atypia.

VI. Spindle cell (sarcomatoid) Carcinoma

They are usually ulcerated and appear as an infiltrative mass or as a polypoid growth in the lip, tongue or other parts of oral cavity. The sarcoma like lesions are usually mixed with squamous cell carcinoma. They may be found as a recurrence of previously diagnosed squamous cell carcinoma. These along with electron microscopic, immunohistochemical and molecular data shows that the sarcomatoid component indicates a metaplastic change of an original epithelial neoplasm. Immunostains help to differentiate between sarcomatiod and sarcoma. The immunostains are epithelial membrane antigen, (EMA) membranous epithelial cadherin and nuclear p63. The sarcoma like component mimic malignant fibrous histocytoma. It may show evidence of specific mesenchymal differentiation along the muscle lines. Hyaline globules is seen in the cytoplasm of larger cells. The prognosis depends on the depth of invasion.

Sarcomatoid tumour of the larynx usually occur as a polypoidal growth[47] and mimic a laryngeal polyp. Microscopically, they have both squamous cell component and sarcoma like component. The sarcomatoid component may mimic as granulation tissue or appear like malignant fibrous histiocytoma, malignant giant cell tumour or osteosarcoma.

Risk Factors

The risk factors of HPV positive HNSCC are similar to the HPV positive cervical squamous cell carcinoma. The patients with HPV positive HNSCC are younger age group with less tobacco and alcohol intake[48].

Molecular profile of HPV positive and HPV negative HNSCC differentiate distinctly and shows the similarity of the HPV positive cervical squamous cell carcinoma. The similarities of the HPV positive cervical carcinoma cases are younger age, age at first intercourse, oral sex, number of sexual partners[49].

Risk factors for HPV-positive and HPV-negative oropharyngeal cancer
HPV-positive oropharyngeal cancers
• Number of oral sex partners
• Many vaginal sex partners
• Young age at first sexual contact
• Anogenital warts
HPV-negative oropharyngeal cancers
• Consumption of nicotine
• Consumption of alcohol
• Older age
• Poor oral hygiene

Table 1.

Although many HNSCC have multifaceted risk factors, tobacco, alcohol and sexual behavior are the major risk factors of the HNSCC. Various international health agencies have conclusively reported that tobacco use is the main cause of 8 major carcinomas in maximum 13 sites which include HNSCC. Mortality rate of tobacco users is high when compared with never smokers. The risk of tobacco causing carcinoma increases with increase in consumption of tobacco[50].

Study reveals that most of the HNSCC patients are alcohol users. There is no study to support alcohol and tobacco use as the independent risk factor of the HNSCC. The level of alcohol consumption is taken as factor when

considering alcohol as one of the risk factors of HNSCC. There is no such study to report with daily alcohol use, but the reasonable to assume, if increase in alcohol use may lead to risk of HNSCC [51].

The combination of tobacco and alcohol use has been reported as increased risk of HNSCC. When high use of alcohol and tobacco excerpt the synergetic effect on increasing the risk of HNSCC [52].

Viruses like herpes group, adenoviruses and HPV influence in the molecular events governing the cell cycles. Cellular oncogenes, also known as proto-oncogenes, acquire their transforming properties or become activated by gene amplification, point mutations, and gene rearrangements[53]. HPV and herpes group of viruses are now considered as the synergistic viruses which are involved in many HNSCC. More than 100 genotypes have been isolated from benign and malignant neoplasms. The most isolated genotypes are HPV 16 and HPV 18, they are also found in normal oral mucosa. HPV infection HNSCC has genetic alteration of influenced by HPV oncoproteins E6 and E7 which inactivate the tumor suppressing gene[54].

HPV oncoproteins E6 and E7 plays major role in immunocompetence, which alters the intercellular immune mechanism. The HPV oncoproteins are capable of binding with many human gene products, particularly the gene deregulating control of cell proliferation and differentiation[55].

HPVs and molecular mechanisms of HPV-induced carcinogenesis

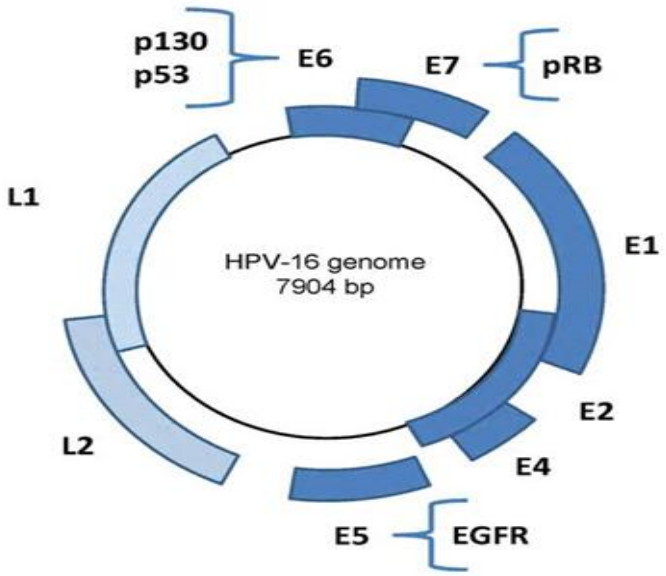

Fig 2. HPV-16 Genome

Human papilloma virus is a Double stranded DNA virus, which is episomal. There are 200 types of known HPV, only few are cancer causing virus. Most of the HPV are infecting the human epithelial cells and connected with different anatomic site preference non-mucosal and cutaneous epithelium[56]. International Agency of Research on Cancer classified HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 as carcinogenic and type 68 as probably carcinogenic to humans[57]. These HPV types are more responsible for the cervical cancer and other sites. Further the HPV is sub-divided into low risk and high risk by the ability of the virus to transform into the host cell. HPV 16, 18, 31, 45 subtypes are categorized as high risk types which is responsible for the cervical cancer and HPV 6, 11 types are low risk subtypes[58].

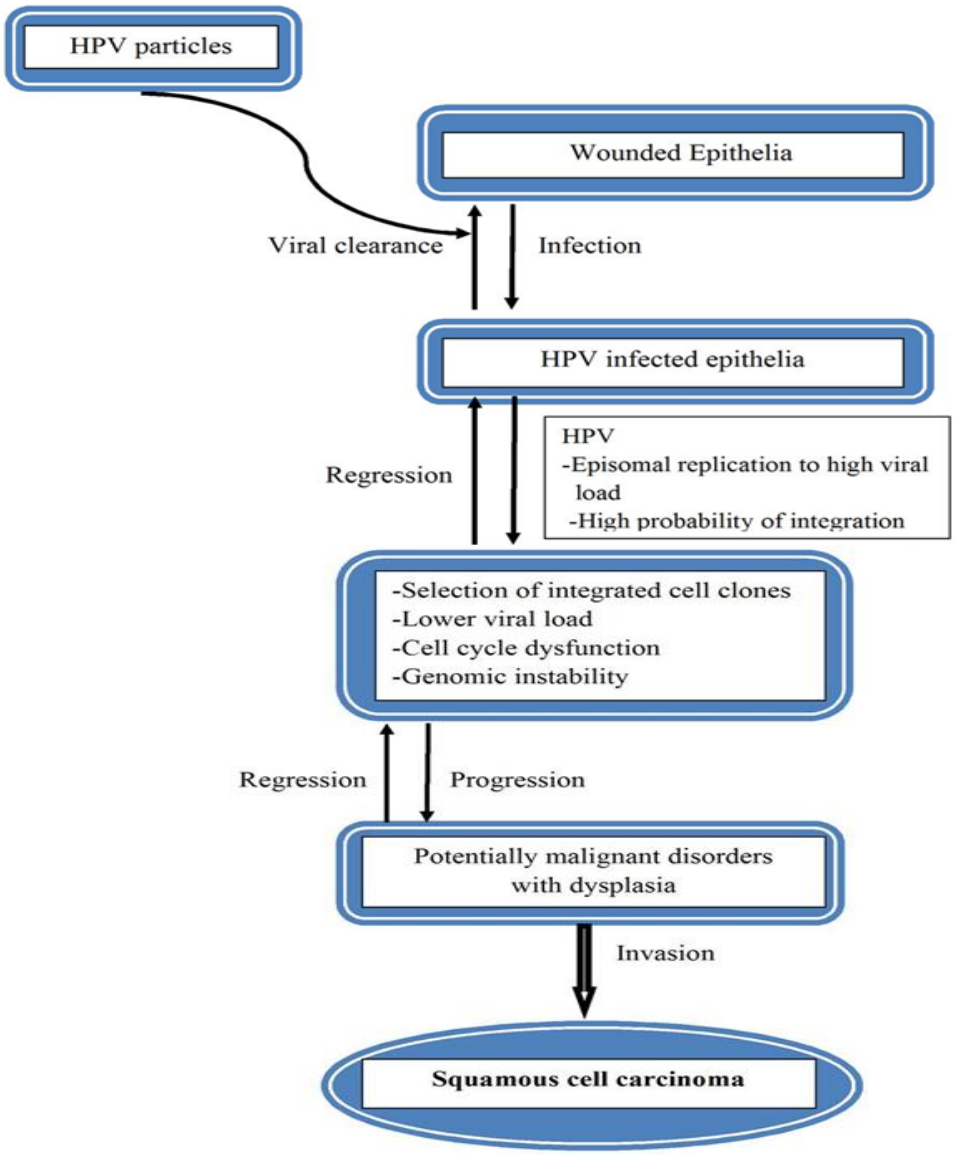

Fig 3. Schematic presentation of the events involved in cancer development after HPV infection

Highest risk	16, 18, 31, 45
Other high risk	33, 35, 39, 51, 52, 56, 58, 59
Probably high risk	26, 53, 66, 68, 73, 82

Table 2. High risk HPV types

The double stranded HPV genome consists of approximately 8000 pairs, divided into 3 regions: a noncoding “long control region” (LCR) regulating gene expression and replication, and 2 protein-coding regions, the early (E1-E7) region coding proteins required for gene expression, replication and survival, and the late (major L1 and minor L2) region coding capsid proteins involved in packaging of viral genome and virus release[59]. The early genes encode 3 viral oncoproteins E5, E6 and E7. E6 and E7 plays a major role both benign proliferation and malignant transformation. E6 and E7 of HPV 16 (and of the other HR types) are able to induce degradation of the tumor suppressor protein p53and pRb via the ubiquitin pathway[60]. The cellular protein E6AP binds with E6, this complex E6/E6AP accountable for the ubiquitination and proteasomal degradation of p53 protein. The p53 plays a role of safeguarding the cell, but there would be a malfunction of p53 in many human malignancies.

This is reported as p53 mutations, happen in HPV infection carcinoma p53 is degraded by E6 oncoproteins[61]. The step toward malignant formation is chromosomal instability of the cells expressing HPV 16 E6 oncoproteins. HPV E7 binds with transcription factor E2F and inactivates the pRb through proteasomal degradation, which controls the G1 S phase transition of cell cycle progress, apoptosis, mitosis and differentiation. Cyclin A and Cyclin E will be activated after E2F release, which leads the entry of cells into G1 S phase. The inactivation of pRb leads to the increased induction of $p16^{INK4a}$. Tumor suppressor $p16^{INK4a}$ is used as biomarker for HPV positive infection lesions and cancer. Most of the HPV positive HNSCC show $p16^{INK4a}$ over

expression. In the HPV negative HNSCC, tobacco and alcohol usage lead to mutational loss of $p16^{INK4a}$ and p53 genes. P16 oncogenic effect depends on the inhibition of CDK4/CDK6 in Rb inactivated cancer cells. CDK4/CDK6 substrate present in cells with inactivated Rb, when phosphorylated may cause cell death[62,63,64] .

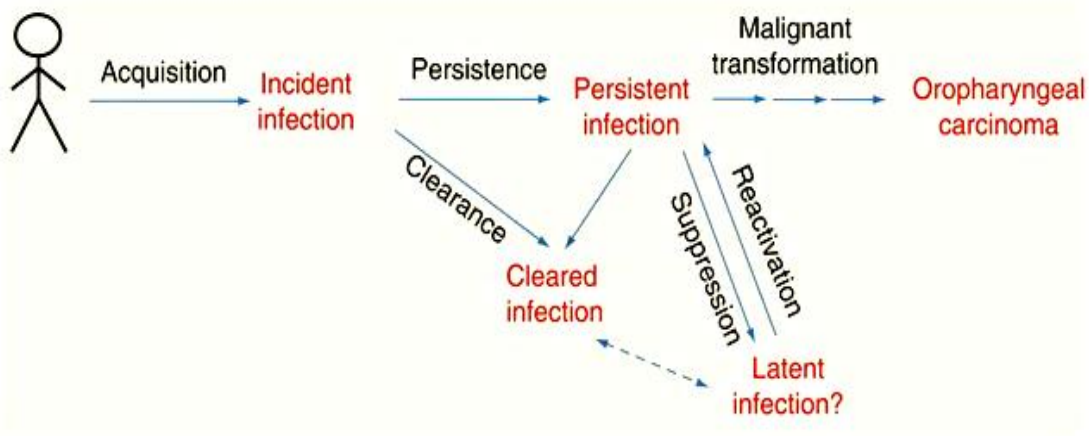

Fig 4 . Proposed oral HPV natural history

Diagnostic (Detection) Methods for HPV-Positive Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinoma

HPV Positive HNSCC do not require the status of HPV evaluation. Many institutions doing this as the standard care for the HPV Positive HNSCC. HPV induced HNSCC have different tumor entity and histopathological features and improved prognosis. Nevertheless, heterogeneity in both biological and clinical behaviour among HPV positive cases has been observed in many studies. This is due to the different viral gene expression and viral load and highlights the need of HPV evaluation in tumor using an algorithm that detects the biologically active HPV, and identifies the

cases with improved outcome[65,66,67,68]. There is no consensus on the optimal way to identify HPV positive HNSCC. Molecular detection is the Gold Standard for detection of HPV DNA in tissues and exfoliated cells samples. Southern blot test is one of the oldest methods to detect HPV DNA with low false positive rates and high sensitivity, and also has the ability to identify the HPV subtypes.

Southern blot is not suited for routine clinical use due to time intensive and require large quantities of cellular DNA. Detection of p16 expression using Immunohistochemistry (IHC) has less specificity than southern blot test[69,70,71]. Polymerase chain reaction (PCR) is used to detect the p16 expression as well as the genetic material related to HPV within the small tumor samples. PCR leads to false positive results, high sensitivity to viral genome that may be present in oral tissues but that are not related to cancer. In situ hybridization (ISH) is used to detect the HPV subtypes; it has lower specificity than Southern blot test. The presence of HPV specific antibodies in serum has also been associated with the risk of developing HNSCC [72,73].

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Detection of HPV infection			
		Advantages	**Disadvantages**
DNA	**PCR**	High sensitivity Type-overlapping and type specific Formalin fixed tissues acceptable	Risk of contamination False positive results possible
	In situ Hybridisation	Morphologic analysis in tissue slides	Low sensitivity Type specific probes
	Southern Blot	Type specific Integration visible High specificity	Low sensitivity High DNA consumption
RNA	**Real-time PCR**	High sensitivity and specificity Detection of biologic activity	Complex procedure Fresh tissue necessary
	In situ Hybridisation	Morphologic detection in tissue slides	Low sensitivity
Protein	**E6/E7**	Easy procedure Detection of biologic activity in fixed tissues possible	Fluctuant sensitivity and specificity
	p16		

Table 3.

p16 ^{INK4a} ImmunoHistoChemistry (IHC)

The standard test for detecting HPV involvement in tumor is detection of Cyclin-dependent kinase inhibitor 2A (p16^{INK4a} protein) over expression, which is a suitable surrogate biomarker and used as the first level of clinical assessment. The over expression of tumor suppression gene p16^{INK4a} is during the immortalization of the host cells, high risk HPV E7 results in downregulation of Rb thus freeing E2F. Free E2F results in upregulation of p16^{INK4a}. The quick, inexpensive method of detection of p16^{INK4a} has become the standard for clinical assessment but it should be noted that all p16^{INK4a}

positive tumors are not HPV positive. 10% to 20% of $p16^{INK4a}$ positive HNSCC have shown no evidence of HPV infection. Few studies reported that no over expression of $p16^{INK4a}$ was observed in patients who were positive for HPV DNA and mRNA. Furthermore, a study demonstrated an over expression of $p16^{INK4a}$ in young patients with oral tongue Squamous Cell Carcinoma without evidence of HPV infections. One more study also showed that OPSCC patients who were $p16^{INK4a}$ -positive and seronegative for HPV antibodies had poor prognosis[73-76]. From these reports, we infer that $p16^{INK4a}$ over expression alone is not sufficient to correctly identify HPV infection in HNSCC. But, $p16^{INK4a}$ IHC alone is used as a choice by many clinicians, because it is widely studied and cost effective with clear staining interpretation guidelines. In the future, DNA and RNA based methods for HPV identification will become necessary along with $p16^{INK4a}$ IHC.

HPV DNA Detection

HPV DNA Polymerase Chain Reaction (PCR)

The high sensitive and cost effective method of detection of HPV DNA strains, targeting to amplify fragments of the conserved HPV L1 gene are commonly used to detect the broad spectrum of HPV such as MY09/MY11 and GP5/GP6 primer pairs. The MY09/MY11 primer pair is synthesized with several degenerate nucleotides in each primer and is thus a mixture of 25 primers capable of amplifying a wide spectrum of HPV types[76,77,78]. Tumorigenic E6 and/or E7 sequence, which is retained by infected cells through viral genome integration, may prove to be more sensitive in the

detection of HPV. Standard PCR techniques have low specificity and do not allow for a distinction between tumor-derived or healthy stroma-derived HPV. Furthermore, PCR techniques are not able to distinguish between episomal and integrated HPV DNA, thus decreasing the ability to detect clinically relevant infection[79,80].

HPV DNA In- Situ Hybridisation (ISH)

A method to detect HPV DNA in tumor samples. It has a high specificity compared to PCR method. It detects and identifies HPV in topographical relationship with their pathological lesions. The appearance of precipitate within the nuclei of epithelial cells indicates HPV presence, which can be seen microscopically. Also, integrated and episomal HPV DNA are distinguished by the presence of punctuate or diffuse signals, respectively. HPV DNA presence defected by ISH, significantly correlated with $p16^{INK4a}$ IHC. Despite the high specificity of this method (100%), sensitivity is low (86%). Therefore, it gives 13-41% false negative rate in HNSCC [81,82,83].

HPV RNA Detection

HPV RNA Polymerase Chain Reaction (PCR)

PCR method of detecting mRNA is a good approach of detecting HPV infection than DNA detection. PCR mRNA detection method provide more clinically relevant evidence of HPV infection than DNA detection. The mRNA expression from episomal HPV DNA indicates the viral oncogenic transcript is crucial in tumor initiation and progression. The over expression E6 and E7 has been detected in HPV positive, tumor samples. The E6/E7

mRNA expression has been shown to highly in HPV positive tonsillar squamous cell carcinoma and lower in oropharyngeal. The instability of the RNA and suboptimal preservation of the biopsy samples using formalin-fixed paraffin-embedded (FFPE) analyses, the methods discussed thus far for HPV transcript detection rely on the analysis of fresh-frozen tissue in research laboratories, therefore hampering the translation to routine clinical diagnostic test. High risk HPV E6/E7 mRNA ISH has been developed as a potential detection tool in FFPE tissues[84,85,86].

HPV RNA In- Situ Hybridisation (ISH)

Recently, high risk HPV E6/E7 mRNA ISH is developed as a potential detection tool in FFPE tissues. In a study of Oropharyngeal squamous cell carcinoma patients, RNA ISH was positive in 99.3% of $p16^{INK4a}$ – positive tumors and had a better sensitivity in HPV detection than DNA ISH. Schache et al reported sensitivity and specificity of 97% and 93% respectively, in the detection of high risk HPV using RNA ISH in FFPE Oropharyngeal squamous cell carcinoma samples[87,88,89]. Hence, detection of HPV transcripts is highly concordant with active and clinically relevant HPV infection which can be incorporated into the current clinical diagnostic methods.

HPV DNA and RNA ISH which have high specificity is used in combination with P16 IHC or HPV DNA PCR which have high sensitivity are effective in diagnosis of HPV associated HNSCC. Screening tumors with $p16^{INK4a}$ IHC is to be performed first, and if the result is positive, to be followed by HPV – specific test, such as ISH or PCR. This provides evidence

that HPV is in the tumor and that the HPV is transcriptionally active based on P16 over expression and E6/E7 mRNA expression levels[90].

HPV Serology

HPV serology is used to detect the previous and current HPV infection status using IgG in serum samples. It is an important biomarker to know the status of the HPV infection. These serological biomarkers are not site specific biomarker; it can arise due to HPV infection at sites other than oral cavity. The HNSCC patients with HPV positive E6 and E7 seropositive showed a favorable cause of survival compared to seronegative patients[91,92].

HPV Detection in Oral fluid

Recently, the use of biological markers in oral fluids for the detection of HPV associated HNSCC is gaining attention, due to the non invasive and cost effective nature, as well as the proximity to oral tumors, which allows early cancer detection and monitoring of disease progression. Oral fluids may allow detection of HPV and cellular alterations in infected cells, which may aid in early detection and typing of HNSCC tumors[93,94] .

A study of oral exfoliated cells and tumor tissues from HNSCC patients showed significant correlation between HR – HPV detected in oral rinse and high risk HPV types present in tumor tissues. Assessment of HPV genotypes in oral rinse may be predictive of HPV associated HNSCC and HPV infection is a risk factor for HNSCC independent of alcohol and tobacco use[95]. Comparing the use of swabs or scrapes to collect mucosal cells from a limited number of oral sites, use of oral rinse is likely to have sampled the tumor site

and the localised field of HPV infection. Another study utilized real time quantitative PCR (RT-qPCR) to detect HPV 16 E6 and E7 DNA in oral rinses for screening HNSCC. RT-qPCR enables more accurate quantification of HPV DNA copy number present in samples with high sensitivity than non quantitative amplification methods. 45.7% of primary tumor tissues and 32.6% oral rinses from HNSCC patients had detectable HPV 16 DNA[96].

HPV DNA detection in tumor tissues and oral rinse from patients with tumors demonstrated significant correlation ($P<0.001$). A recent prospective study demonstrated that HPV detection in oral rinse was comparable with HPV presence in tumor tissues. Earlier study showed the presence of HPV DNA in tumor tissues and in oral rinse samples and was associated with presence of HPV specific antibodies in sera. There is a feasibility of correlating high risk HPV DNA positivity in oral rinses and HPV- related antibodies in the blood for detection and surveillance of disease progression[97].

The presence of high risk HPV DNA in oral samples may be a strong biomarker for development of OPSCC in high risk groups with premalignant lesions and immuno compromised individuals. A recent study analysed and correlated presence of HPV DNA in oral samples (oral rinses and /or tonsillar swabs) in patients with incidence of HPV positive tonsillar and base of tongue cancer. Presence of HPV DNA in oral samples that is concordant with HPV positivity in tumor samples was detected in 76% and 50% of patients with tonsillar and base of tongue cancer, respectively[98,99] .

Studies to data have demonstrated promising data for the utilization of oral fluid as a valid specimen for detection of high risk HPV. At present, there is only one laboratory based salivary diagnostic test available to detect HPV, by detecting different strains of HPV such as HPV 8,11,16 and 18 via PCR using oral rinse samples. However, the sensitivity and specificity of oral fluid based detection methods lack experimental evidence. It is also important to experimentally validate if the HPV infection is current/active or in the past. Oral fluid based tests to determine HPV infection require further improvements because of the different origins of cells being tested in oral fluid.

Clinical features of HPV-induced Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinomas

HPV positive HNSCC is more likely to be young aged, non-tobacco users, mild to moderate alcohol users. HPV positive HNSCC patients are having multiple sex partners or having oral sexual behavior. Patients with HPV positive HNSCC have high survival rate than HPV negative patients. HPV induced squamous cell carcinoma develops from oropharynx and base of the tongue being more commonly involved in HNSCC [100,101,102]. Tonsillar crypts covered with reticulated epithelium which has contact with immune system may prone to HPV infection and subsequent malignant transformation[103]. There are no specific histologic features to discriminate the HPV induced from non-HPV induced squamous cell carcinoma. Identification of HPV positive and HPV negative HNSCC is important to identifying the specific targets in each subjects and understanding the pathogenesis to provide

the targeted therapies. The morphological characters of HPV driven carcinogenesis, while the prototypic HNSCC is moderately distinguished HPV-induced SCCs are predominantly non-keratinizing SCC often described as poorly differentiated carcinomas or basaloid carcinomas based on the lobular growth of cells with hyperchromatic nuclei, scanty cytoplasm and marked mitotic activity[104,105,106,107] . HPV positive HNSCC have specific clinical presentation of tumor and neck stage. Compared to non-HPV related carcinomas, HPV related carcinomas are diagnosed in an earlier of T-category with a trend for a more advanced N-category[108].

Prognosis of HPV-induced Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinomas

The evidence of HPV induced HNSCC came from various single site retrospective case series studies published in the last decades, shown that HPV positive HNSCC cases particularly the oropharyngeal carcinoma patients treated with radiotherapy, chemotherapy, surgery and combined modality therapy shown better outcome than the HPV negative HNSCC cases [109-112]. These studies shown there is reduction of risk of disease failure when compared HPV positive squamous cell carcinoma with HPV negative patients. The reason behind the HPV positive HNSCC better prognosis than HPV negative cases remains elusive. The overall survival rate of HPV positive cases depend on the younger age at diagnosis, lower tobacco and alcohol use, distinct biology of cancer, reduced risk of secondary tumor or aggressive treatment strategy. The favorable outcome of HPV-induced SCC may be attributable to enhanced sensitivity to treatment due to a wild-type TP53,

allowing an apoptotic response of cancer cells to radiation and chemo radiation[113]. Tobacco use may alter the clinical behaviour of the HPV positive HNSCC adversely affecting the prognosis. The high risk HPV E6 and E7 oncoproteins target tumor suppressor signaling pathways. A major transforming characters of HPV16 E6 is its ability to induce perversion of the tumor suppressor protein p53 via the ubiquitin pathway in transcriptionally active HPV infections, HPV16 E7 inactivates pRb. This event is connected with up-regulation of CDKN2A, which codes for $p16^{INK4a}$. The absence of TP53 gene mutations is significantly associated with better overall survival and $p16^{INK4a}$ positivity, irrespective of HPV status, is also associated with better outcomes[114] .

Management of HPV - Positive Oropharyngeal and Upper Respiratory Tract Squamous cell carcinoma.

Treatment of HPV positive HNSCC is a serious problem, as although there is no clear evidence from randomized, controlled trial to support the reduction of treatment intensity in HPV positive Squamous cell carcinoma. Some researchers argued, that intensive concomitant chemoradiation therapy amounts to overtreatment. HPV positive patients are younger, an multimodality strategy, which may result in high rates of acute and long term severe toxicity would not be suitable for prolonged survival. In this situation, efforts are taken to reduce the toxicity and increase the quality of life in long term, while maintaining the efficacy[115-117] .

In strengthening the treatment for HPV positive squamous cell carcinoma patients, including altered radiation fractionation and the combination of chemotherapy and altered radiation fractionation, patients still have the problem of significant challenge of recurrent or second tumors arising within short period. Locally recurrent tumors may arise from residual neoplastic cells that survive initial treatment, perhaps because of biological parameters that confer radio-resistance or insufficiencies in initial treatment parameters such as radiation dose, volume, fractionation and treatment duration. A treatment de-escalation would be achieved by reducing the total dose of radiotherapy as well as the dose of chemotherapy[118] .

Many researches are mainly based on the reducing the intensity of the radiotherapy or on substituting cis-platinum with cetuximab in concurrent chemotherapy regimens. Furthermore emerging data shown cetuximab in radiotherapy may not be preferable for HPV positive cancers[119,120]. Nevertheless, the optimal treatment for HPV positive HNSCC patients remains uncertain. HPV positive cancers appear more sensitive to chemoradiation as patients with low risk HPV positive oropharyngeal cancers have almost double the overall survival as patients with high risk HPV negative cancers. This benefit in HPV positive patients results from improved locoregional control rather than decreased distant metastasis.

Trans-oral surgery is evolving as a feasible treatment option for early stage Squamous cell carcinoma of the oropharynx. Nominally invasive trans-oral surgery can be performed by transoral laser microsurgery or trans-oral

robotic surgery. The benefits of minimally invasive trans-oral surgery include low morbidity and mortality and good functional outcomes[121].

Prevention of HPV- Positive Head and Neck Squamous Cell Carcinomas

Cancer may arise due to mutations or from exposure to environmental factors. As cancers are quite heterogeneous in nature, with a multitude of mutations, identifying therapies that work on entire tumor cell populations including tumor-initiating or cancer stem cells has been difficult. Current management procedures such as surgery, radiation, chemotherapy which impact the quality of life of the patients. The changing ability of human papillomavirus in the cervix, anogenital tract, and oropharynx suggests that prevention of HPV infection may ultimately prevent HPV associated cervical and head and neck cancers.

Gardasil and Cervarix vaccines are used to prevent most common oncogenic HPV types known to cause cervical cancer. High risk HPV 16 and HPV 18 is prevented by *Cervarix* a bivalent vaccine, *Gardasil* is a quadrivalent which protects against HPV 16 and HPV 18 as well as the low risk HPV 6 and HPV 11 types[122,123]. United States Food and Drug Administration (FDA) recommend vaccination for men and women, between the ages of 9-26 years. HPV infection most likely occurs during this age group. It is important to begin vaccination at the earliest recommended ages, before the start of sexual activity to get maximum protection against HPV infection. Vaccination after the HPV infection does not result in increased protection from the virus or clearance of it. These vaccines utilize virus-like

particles (VLP) formed by self-assembly of the HPV capsid protein L1 to elicit the immune response, as opposed to live or attenuated virus. Antibodies are then produced against the L1-specific to each HPV type included in the vaccine[124].

These vaccines are protective against cervical, vaginal and vulvar cancers and also genital warts. These vaccines are not yet approved by U.S. F.D.A. for HPV infected HNSCCs. This is due to the lack of detailed information on HPV associated HNSCCs required by the F.D.A. As more than 90% of all HNSCCs are HPV 16, these vaccines may be highly efficacious. Use of currently available vaccines following development of cancer, do not provide clinical benefit, because expression of the capsid proteins is lost during transformation. Oncologists can advocate universal HPV vaccination for both boys and girls, a recommendation shared by CDC (Centers for Disease Control and Prevention) and American Academy of Pediatrics.

	Prophylactic vaccine	**Therapeutic vaccine**
Prevent new infection	Yes	No
Treat existing infection	No	Yes
Effector cells	B cells	CD4+ T cells, CD8+ T cells
Humoral (Antibody) response	High	Low
Cell mediated (T-cell) response	Low	High
Vaccine construction	Whole organism- live/ attenuated / killed; Protein subunits	DNA plasmid; Peptides/proteins; Dendritic cell
Targeted antigen	Cell surface viral capsid protein	Intracellular viral protein

Table 4. Differences between Prophylactic and Therapeutic Vaccines

Future directions

Many groups are doing research to better understand the biology of HPV- Positive HNSCC, to improve prevention, screening, diagnosis and identify and validate novel therapeutic targets.

Till now limited information is available about the natural history of oral HPV infection.

Further longitudinal research is needed to better understand the transmission of oral HPV infection, clearance and persistence of the infection. Similar to cervical cancer, persistence of HPV infection may likely prove central for HPV positive HNSCC.

Prophylactic HPV vaccines are having high efficacy for the prevention of anal, cervical, vaginal and vulvar cancer development among those individuals who are not previously exposed to HPV infection.

Direct evaluation of vaccine efficacy against head and neck HPV infection and tumor development is still necessary as there are no published data on this topic.

HPV vaccination as a public health measure against anogenital HPV infection will most probably also have a favorable impact on the frequency of HPV associated HNSCCs.

Biomarker detection in saliva seems to be promising for secondary prevention.

The incorporation of new screening method like HPV detection in oral fluid into the current diagnostic methods will help in early detection and intervention, risk assessment and response to treatment of HNSCC.

Further research has the potential for the development of a screening test similar to Papanicolaou test, which could be used to screen individuals at high risk for developing HPV-associated HNSCC.

HPV-positive HNSCC, after treatment has to be monitored for detection of disease persistence or early disease recurrence.

Salivary rinsing as a possible screening test for recurrence of HPV positive tumors, after treatment has been evaluated. In a study with small number of persons, detection of E6 and E7 copy number by RT-PCR from salivary rinses had a specificity of 50% and sensitivity of 100%. for detection of oropharyngeal cancer recurrence.

However, larger study is needed to determine the value of salivary rinsing as a screening test for tumor recurrence.

In the next few years, we will likely to use HPV tumor status not only for prognostic purpose, but also for selection of treatment approaches.

Further studies will help in implementation of de-escalation of treatment intensity in HPV-Positive HNSCC.

In the near future we may employ significantly different treatments for patients with HPV-positive as compared to HPV negative HNSCC.

Understanding the biology of HPV positive HNSCCs at the molecular level, will provide the ability to rationally personalize therapy to improve therapeutic outcomes.

In the near future, targeted therapy for HPV associated HNSCCs will become possible.

The most important trials going on for targeted therapy are, Extracellular blocking of HER/EGFR, Monoclonal antibodies against EGFR, Intracellular blocking of HER/EGFR network etc.

Immunotherapies targeting HPV E6 and E7 oncoproteins are being developed which will be of benefit in HPV associated Head and neck squamous cell carcinomas.

TNM Staging of Carcinoma of the Oropharynx and Hypopharynx

Primary tumor (T)

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

T_{IS} Carcinoma in situ

Oropharynx

T1 Tumor 2 cm or less in greatest dimension

T2 Tumor more than 2 cm but not more than 4 cm in greatest dimension

T3 Tumor more than 4 cm in greatest dimension or extension to lingual surface of epiglottis.

T4a Moderately advanced local disease

Tumor invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible

T4b Very advanced local disease

Tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base, or encases carotid artery.

Hypopharynx

T1 Tumor limited to one subsite of hypopharynx and/or 2 cm or less in greatest dimension

T2 Tumor invades more than one subsite of hypopharynx or an adjacent site, or measures more than 2 cm in greatest dimension without fixation of hemilarynx.

T3 Tumor more than 4 cm in greatest dimension or with fixation of hemilarynx or extension to esophagus.

T4a Moderately advanced local disease

Tumor invades thyroid/cricoid cartilage, hyoid bone, thyroid gland, or central compartment soft tissue

T4b Very advanced local disease

Tumor invades prevertebral fascia, encases carotid artery, or invades mediastinal structures.

Regional Lymph nodes (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension.

N2 Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension, or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension, or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.

N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension.

N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension.

N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.

N3 Metastasis in a lymph node, more than 6 cm in greatest dimension

Distant Metastasis (M)

M0 No distant metastasis

M1 Distant metastasis

Anatomic Stage / Prognostic groups

Stage 0	$T_{IS}N0M0$
Stage I	T1N0M0
Stage II	T2N0M0
Stage III	T3N0M0
	T1N1M0
	T2N1M0
	T3N1M0
Stage IVA	T4aN0M0
	T4aN1M0
	T1N2M0
	T2N2M0
	T3N2M0
	T4aN2M0
Stage IVB	T4b Any N M0

Any T N3 M0

Stage IVC Any T AnyN M1

TNM Staging of Carcinoma of the Larynx

Primary tumor (T)

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

T_{IS} Carcinoma in situ

Supraglottis

T1 Tumor limited to one subsite of supraglottis with normal vocal cord mobility

T2 Tumor invades mucosa of more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis (e.g. mucosa of base of tongue, vallecula, medial wall of pyriform sinus) without fixation of the larynx.

T3 Tumor limited to larynx with vocal cord fixation and/or invades any of the following: post cricoids area, pre-epiglottic tissues, paraglottic space, and or minor thyroid cartilage erosion (e.g. inner cortex)

T4a Moderately advanced local disease

Tumor invades through the thyroid cartilage and/or invades tissues beyond the larynx (e.g. trachea, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid or esophagus)

T4b Very advanced local disease

Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.

Glottis

T1 Tumor limited to the vocal cord(s) (may involve anterior or posterior commissure) with normal mobility.

T1a Tumor limited to one vocal cord

T1b Tumor involves both vocal cords

T2 Tumor extends to supraglottis and/or subglottis, or with impaired vocal cord mobility.

T3 Tumor limited to larynx with vocal cord fixation, and/or invade paraglottic space and/or minor thyroid cartilage erosion (e.g. inner cortex)

T4a Moderately advanced local disease

Tumor invades through the thyroid cartilage and/or invades tissues beyond the larynx (e.g. trachea, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid or esophagus)

T4b Very advanced local disease

Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.

Subglottis

T1 Tumor limited to the subglottis

T2 Tumor extends to vocal cord(s) with normal or impaired mobility

T3 Tumor limited to larynx with vocal cord fixation

T4a Moderately advanced local disease

Tumor invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx (e.g. trachea, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid or esophagus)

T4b Very advanced local disease

Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.

Regional Lymph nodes (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension.

N2 Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension, or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension, or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.

N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension.

N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension.

N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.

N3 Metastasis in a lymph node, more than 6 cm in greatest dimension

Distant Metastasis (M)

M0 No distant metastasis

M1 Distant metastasis

Anatomic Stage / Prognostic groups

Stage 0 $T_{IS}N0M0$

Stage I T1N0M0

Stage II T2N0M0

Stage III T3N0M0

	T1N1M0
	T2N1M0
	T3N1M0
Stage IVA	T4aN0M0
	T4aN1M0
	T1N2M0
	T2N2M0
	T3N2M0
	T4aN2M0
Stage IVB	T4b Any N M0
	Any T N3 M0
Stage IVC	Any T AnyN M1

MATERIALS AND METHODS

Study Place:

Department of Pathology, Chengalpattu Medical College and Hospital, Chengalpattu.

Study Design:

The present cross-sectional study was a prospective study conducted in the Department of Pathology during the period of June 2014 to August 2015 . Ethical clearance for the study was obtained from the Institutional Ethics Committee of Chengalpattu Medical College, Chengalpattu.

A total sample of 60 cases of HNSCC were analyzed during the period of June 2014 to August 2015.

Study Population

Inclusion Criteria

Tissue blocks of patients who are diagnosed as Oropharyngeal and Upper Respiratory Tract squamous cell carcinoma by biopsy.

Exclusion Criteria:

Tissue blocks of patients who are diagnosed as Oropharyngeal and Upper Respiratory Tract squamous cell carcinoma by biopsy and underwent Radiotherapy or Chemotherapy.

During the period of June 2014 to August 2015, as per the inclusion and exclusion criteria, biopsies received in the Department of Pathology were included. History written in the histopathology request form were recorded on predesigned and pretested proforma (Annexure I).

MATERIALS USED

Tissue sections prepared from paraffin embedded formalin fixed tissues

Haematoxylin and eosin staining kit

$p16^{INK4a}$ monoclonal antibody kit

Positive control

Negative control

Method:

- Blocks and slides of 60 patients in which histopathological examination of hematoxylin and eosin stained sections of biopsy from Oropharyngeal and Upper Respiratory Tract sites confirmed as squamous cell carcinoma were taken up for the study.

- Immunohistochemistry was performed on the tissue sections taken from the blocks of the cases confirmed as squamous cell carcinoma.

Immunohistochemistry Procedure

1. 4µ thick sections were cut from formalin fixed paraffin embedded tissue samples and transferred to gelatin-chrome alum coated slides.

2. The slides were incubated at 58ºC for overnight.

3. The sections were deparaffinized in xylene for 15 minutes x 2 changes.

4. Rehydrated through descending grades of alcohol as follows :

   Absolute alcohol x 2 changes 5 minutes each

   90% alcohol x 5 minutes

   Washed in distilled water 2 changes, 2 minutes each

5. Heat induced antigen retrieval was done with microwave oven at 150 degree celsius with citrate buffer (pH 6.0) for 15 to 20 minutes.

6. Then cooled for 10 minutes.

7. Washed in distilled water 2 changes, 2 minutes each.

8. Washed in TBS for 2 minutes.

9. Endoperoxidase blocking was done by adding hydrogen peroxide on the section and kept for 5 minutes.

10. Washed in the wash buffer for 2 minutes twice.

11. Primary antibody $p16^{INK4a}$ (Mouse monoclonal, Clone (G175-405) ; prediluted) was added and kept for 30 minutes in a moist chamber.

12. Then washed in wash buffer 2 minutes 2 times each.

13. Poly excel target binder reagent was added and kept for 15 minutes.

14. Washed in two changes of buffer 2 minutes each.

15. Poly excel HRP (Horse Radish Peroxidase) was added and incubated for 15 minutes.

16. Washed with buffer – 2 minutes, 2 changes.

17. Working DAB chromogen (1ml DAB buffer + 1 drop chromogen, mix well) was added and kept for 2-5 minutes.

18. Then washed in distilled water.

19. Counter stained with hematoxylin for 30 seconds.

20. The slides were washed in running tap water for 3 minutes.

21. The slides were air dried, cleared with xylene and mounted with DPX.

- Positive control included block sections of known $p16^{INK4a}$ positive cases.

- Negative control included Primary antibody replaced with PBS and normal oral tissue.

- Immunostained sections were reviewed and a strong nuclear as well as cytoplasmic staining was considered as positive reaction, as described by Klaes et al[125].,

- Distribution of $p16^{INK4a}$ positivity were scored as negative (<1% cells positive), sporadic (<5% cells positive), focal (<25% cells positive) and diffuse (>25% cells positive).

Data Collection

H &E stained sections and immunostained sections were assessed using light microscope.

Observations:

History **:** Smoking/Alcohol

Site of the lesion **:**

Histopathological confirmation and grading of H&E stained section :

HNSCC Grade 1

HNSCC Grade 2

HNSCC Grade 3

$p16^{INK4a}$ expression

Negative

(<1% cells positive)*

Positive

Sporadic (<5% cells positive)*

Focal (<25% cells positive)*

Diffuse (>25% cells positive)*

*As described by Klaes et al[125] .

Statistical analysis:

Datas obtained were coded and entered into the Microsoft excel spread sheet (Annexure II). Datas were compared between groups using Pearson Chi-square or Fisher's exact tests ($p<0.05$). All statistical analysis were performed using SPSS statistical software version 11. Charts were prepared using Microsoft excel 2007.

OBSERVATION AND RESULTS

In the present study 75% of the cases of HNSCC were above 50 years of age (Table 5.). However none of the cases were observed below 22 years of age (Table 6.). The beginning age for $p16^{INK4a}$ positive HNSCC cases in our study is 22 years and is 44 years for $p16^{INK4a}$ negative cases. Among the 15 HNSCC cases below 50 years of age, 14 cases (93.33%) were $p16^{INK4a}$ positive. Among the HNSCC cases above 50 years of age 93.33% were positive for $p16^{INK4a}$. The range of the age group is much wider in the $p16^{INK4a}$ positive cases. There is no difference in the mean age among the HNSCC $p16^{INK4a}$ positive and negative groups. The median age for $p16^{INK4a}$ negative cases is slightly lower (58 years)

Age group (years)	**Total n=60**	**HNSCC**				
		$P16^{INK4a}$ +ve n=56		**$P16^{INK4a}$-ve n=4**		**P value**
		n	**%**	**n**	**%**	
Age≤50	15	14	93.33	1	6.67	1.000
Age>50	45	42	93.33	3	6.67	

Fisher's exact test

Table 5. Age distribution of patients with HNSCC

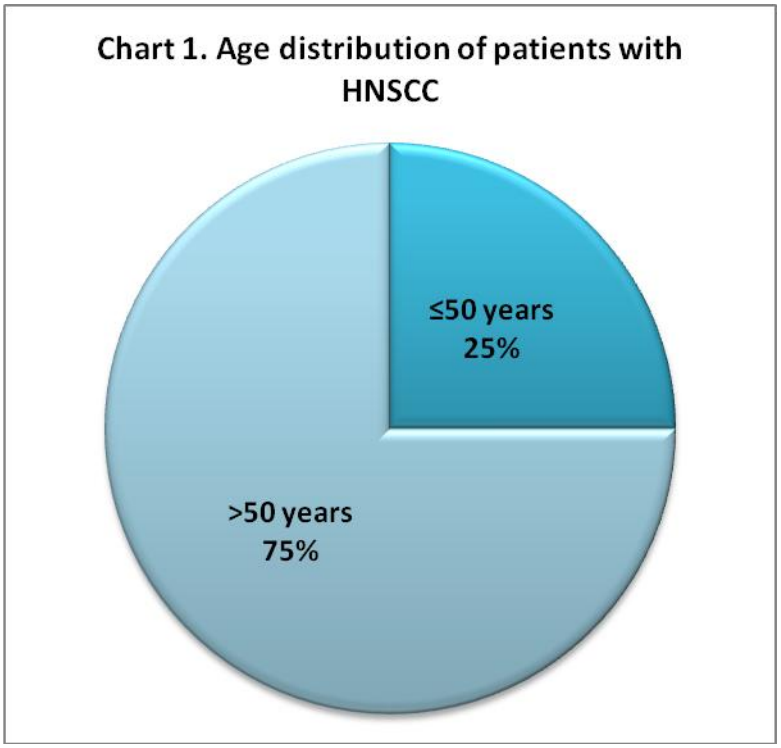


Chart 1. Age distribution of patients with HNSCC
≤50 years
25%
>50 years
75%

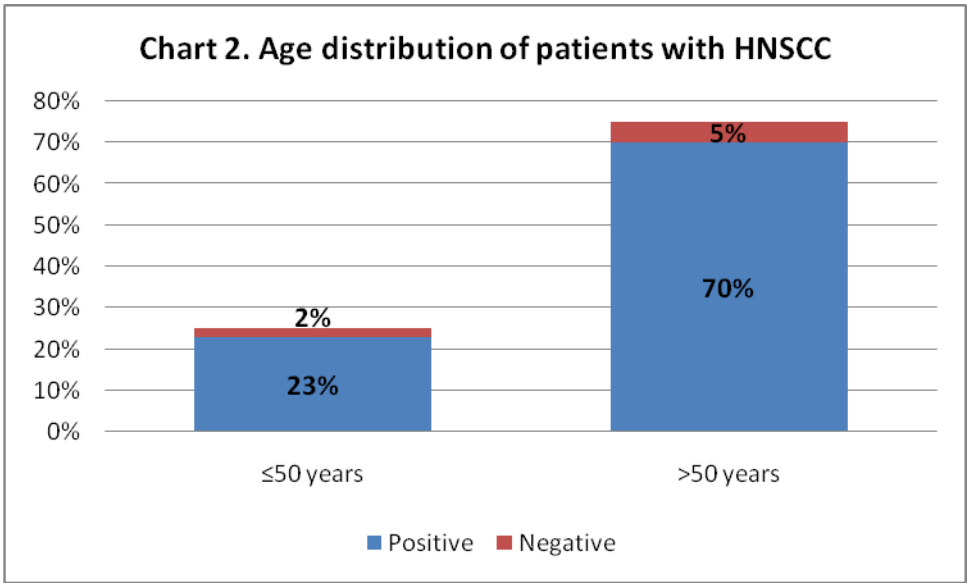


Chart 2. Age distribution of patients with HNSCC
80%
70%
60%
50%
40%
30%
20%
10%
0%
2%
23%
5%
70%
≤50 years
>50 years
Positive
Negative

Age group (years)	HNSCC		
	All patients n=60	**P16^{INK4a} +ve n=56**	**P16^{INK4a} -ve n=4**
Range	22-83	22-83	44-75
Mean±SD	58.68±12.83	58.68±12.94	58.75±12.91
Median	60	60	58

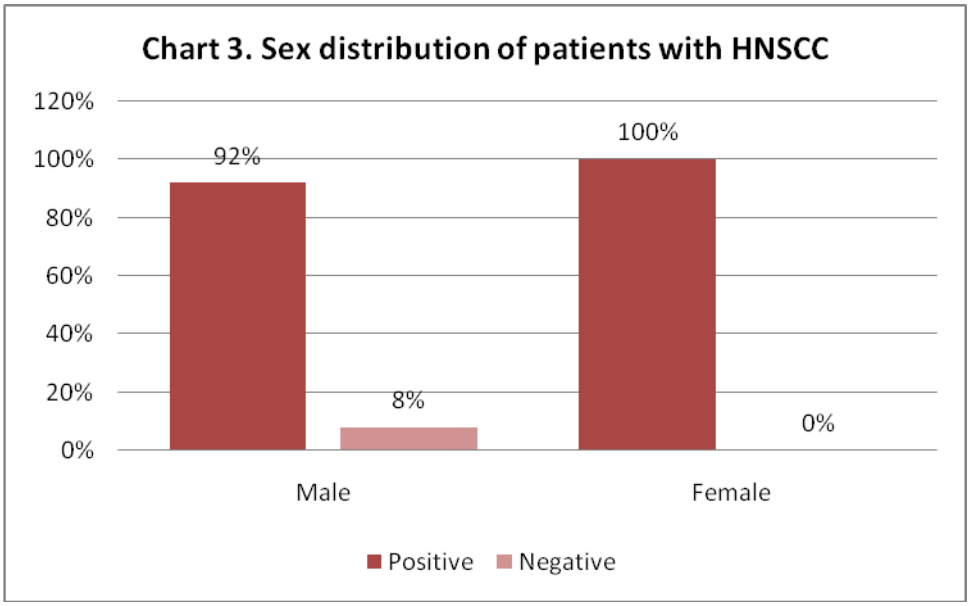
Table 6. Age distribution of patients with HNSCC

In the sex distribution 85% of the HNSCC cases were male and 15% of the cases were female (Table 7.). All the female cases 15 (100%) were p16^{INK4a} positive. 47/51 male cases (92.16%) were p16^{INK4a} positive. 5/9 female cases are ≤50 years and all are p16^{INK4a} positive. 10/51 male cases are ≤50 years and 90% of which are p16^{INK4a} positive. There is no much difference between p16^{INK4a} positive cases in the ≤50 years and >50 years age groups (Table 8.).

Gender	Total n=60	HNSCC				P value
		P16^{INK4a} +ve n=56		**P16^{INK4a} -ve n=4**		
		n	**%**	**n**	**%**	
Male	51	47	92.16	4	7.84	1.000
Female	9	9	100	0	0	

Fisher's exact test

Table 7. Sex distribution of patients with HNSCC



Gender	Total =60	HNSCC											
		Age≤50 years						Age>50 years					
		Total =15	P16^{INK4a} +ve n=14		P16^{INK4a} -ve n=1		P value	Total= 45	P16^{INK4a} +ve n=42		P16^{INK4a} -ve n=3		P value
			n	%	n	%			n	%	n	%	
Male	51	10	9	90	1	10	1.000	41	38	92.68	3	7.32	1.000
Female	9	5	5	100	0	0		4	4	100	0	0	

Table 8. Sex distribution of patients with HNSCC in relation to age.

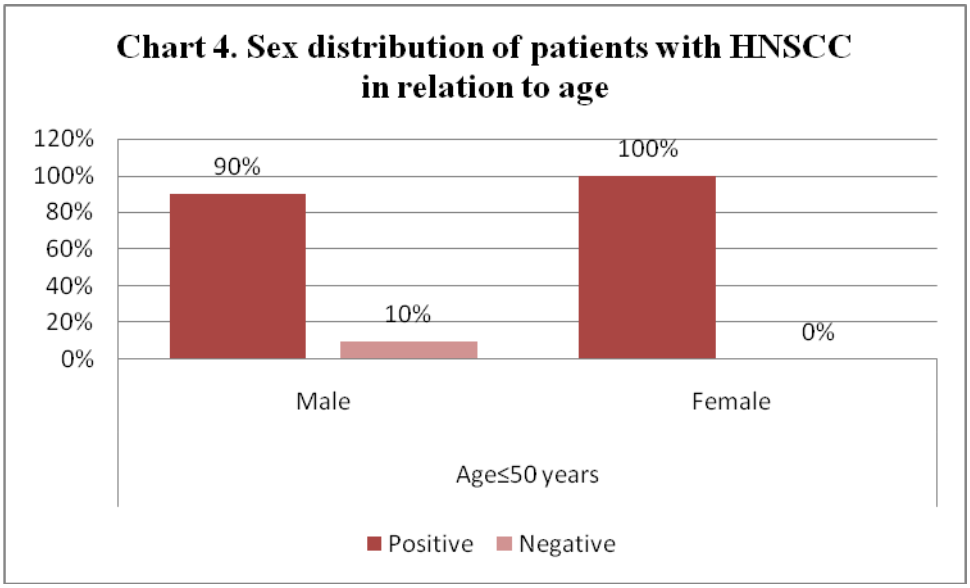


Chart 4. Sex distribution of patients with HNSCC in relation to age
120%
100%
80%
60%
40%
20%
0%
90%
10%
100%
0%
Male
Female
Age≤50 years
Positive
Negative

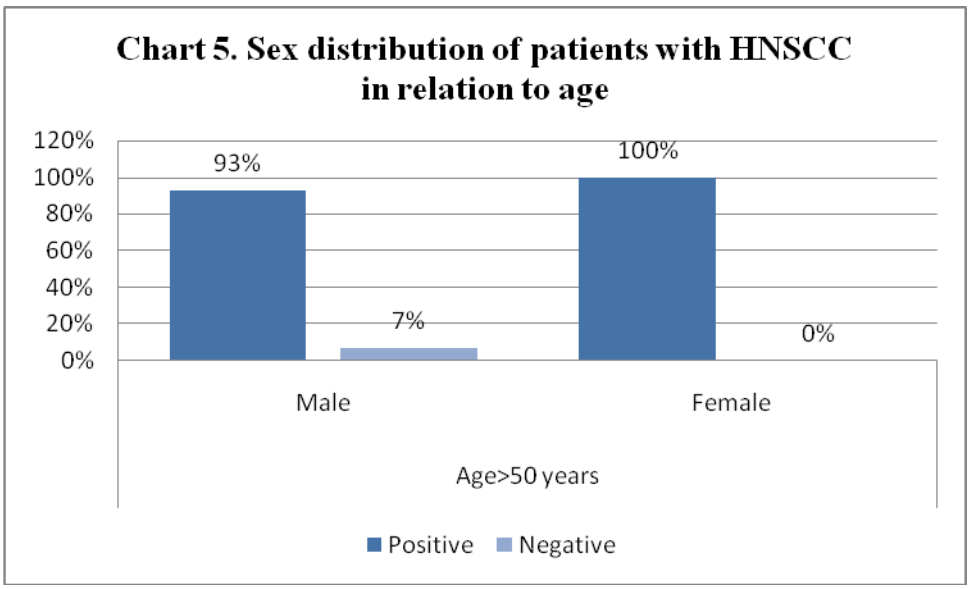


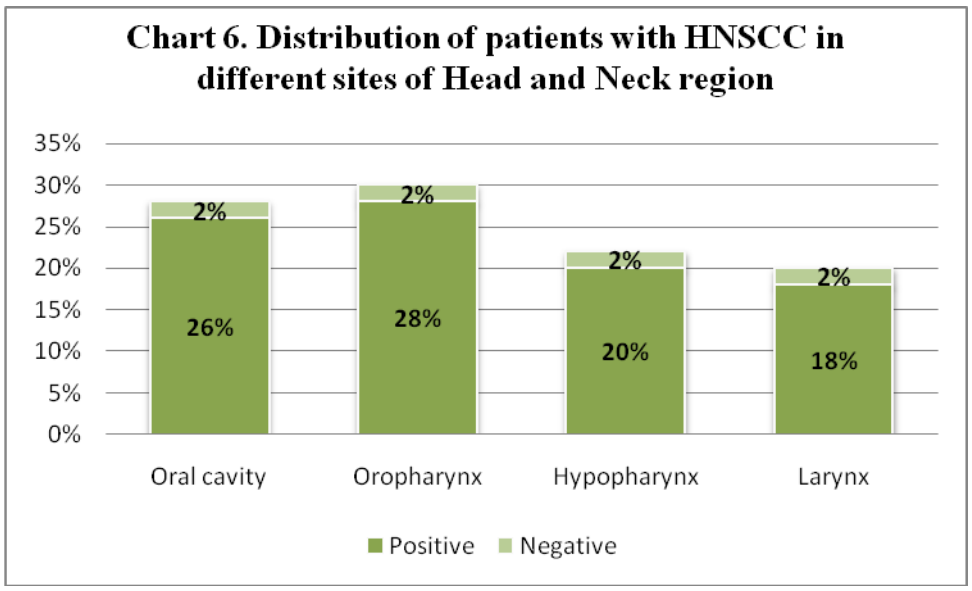
Chart 5. Sex distribution of patients with HNSCC in relation to age
120%
100%
80%
60%
40%
20%
0%
93%
7%
100%
0%
Male
Female
Age>50 years
Positive
Negative

Oropharynx was the most common site involved in the HNSCC (30%), followed by oral cavity, hypopharynx and larynx(Table 9, Chart 6). In our study we did not receive biopsy from nasopharynx. The percentage of $p16^{INK4a}$ negative cases were similar in the different sites of HNSCC.

Site of the lesion	Total n=60	HNSCC				P value
		$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		
		n	%	n	%	
Oral cavity	17	16	94.12	1	5.88	0.988
Oropharynx	18	17	94.44	1	5.56	
Hypopharynx	13	12	92.30	1	7.70	
Larynx	12	11	91.67	1	8.33	
Total	60	56	93.33	4	6.67	

Pearson Chi Square test

Table 9. Distribution of patients with HNSCC in different sites of Head and Neck region

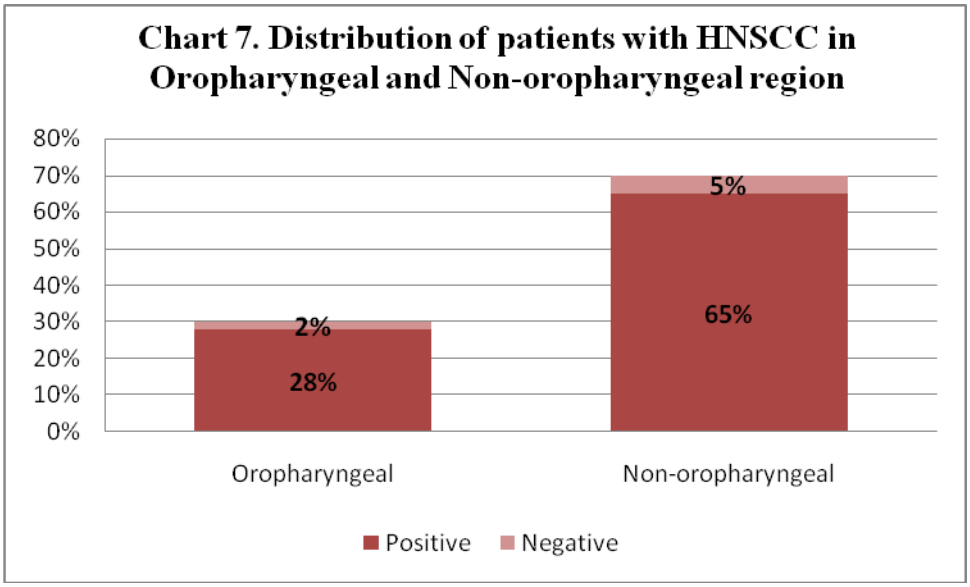


Comparing the oropharyngeal and non-oropharyngeal squamous cell carcinomas, $p16^{INK4a}$ positive cases are 94.44% in the oropharyngeal cases and it is 92.86% in the non-oropharyngeal cases. (1.58% higher in the oropharyngeal squamous cell carcinomas) (Table 10.).

Site of the lesion	Total n=60	HNSCC				P value
		$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		
		n	%	n	%	
Oropharyngeal	18	17	94.44	1	5.56	1.000
Non-oropharyngeal	42	39	92.86	3	7.14	

Fisher's exact test

Table 10. Distribution of patients with HNSCC in Oropharyngeal and Non-oropharyngeal region

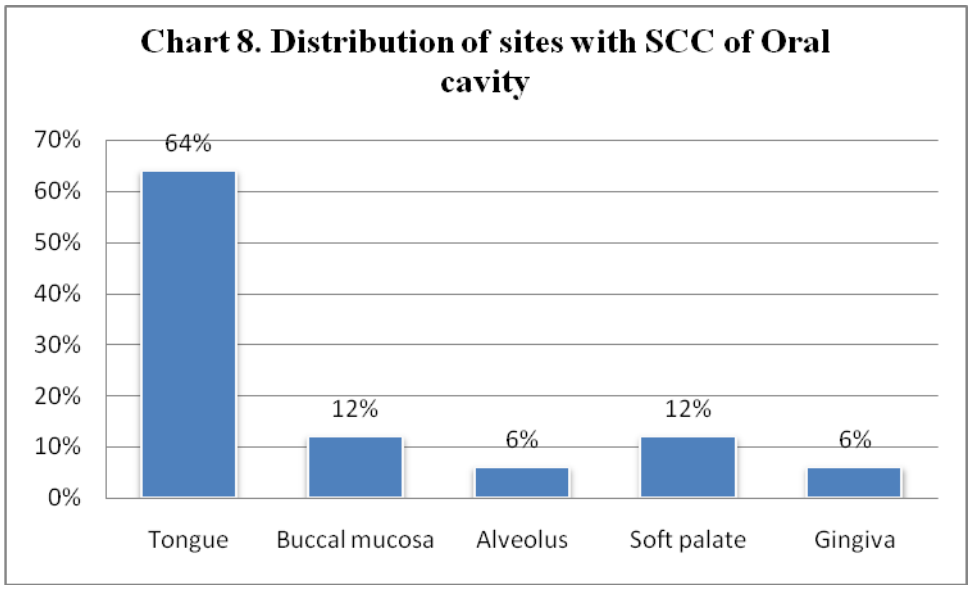


Among the oral cavity squamous cell carcinomas, tongue was the most common site involved (64%), followed by buccal mucosa, soft palate, alveolus and gingiva. 90.91% of the tongue squamous cell carcinomas are $p16^{INK4a}$ positive (Chart 8.).

Site of the lesion	Total n=17	Oral cavity SCC				P value
		$P16^{INK4a}$ +ve n=16		$P16^{INK4a}$ -ve n=1		
		n	%	n	%	
Tongue	11	10	90.91	1	9.09	0.965
Buccal mucosa	2	2	100	0	0	
Alveolus	1	1	100	0	0	
Soft palate	2	2	100	0	0	
Gingiva	1	1	100	0	0	

Pearson Chi Square test

Table 11. Distribution of sites with SCC of Oral cavity

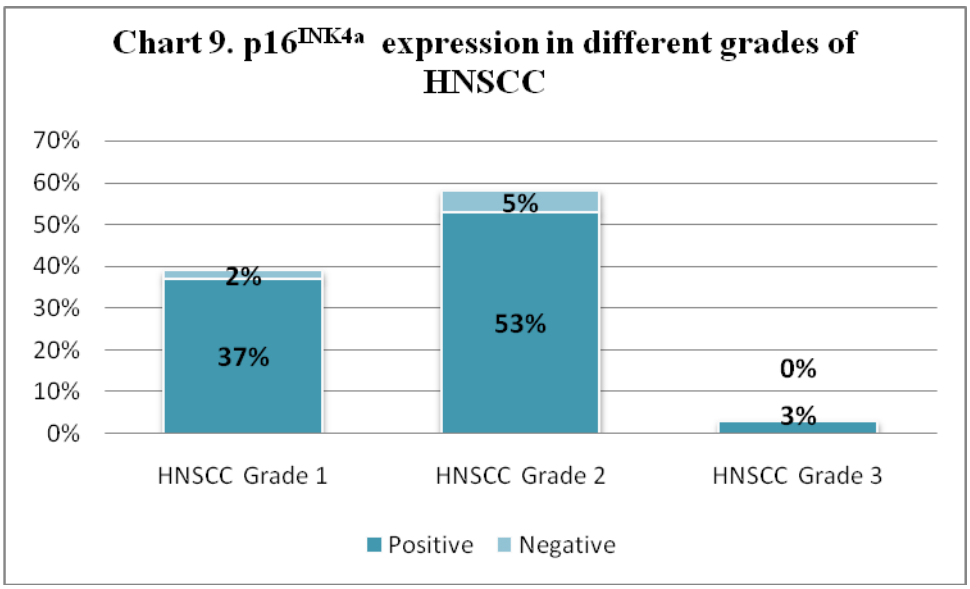


Majority of HNSCC cases in our study were of grade 2 (35/60 cases; 58%), followed by grade 1 (24/60 cases; 39%) and grade 3 (2/60; 3%) on the histopathological grading after Haematoxylin and Eosin staining (Table 12, Chart 9).

Tumour differentiation	Total n=60	$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		P value
		n	%	n	%	
HNSCC Grade1	23	22	95.65	1	4.35	0.761
HNSCC Grade2	35	32	91.43	3	8.57	
HNSCC Grade3	2	2	100	0	0	

Pearson Chi Square test

Table 12. $p16^{INK4a}$ expression in different grades of HNSCC



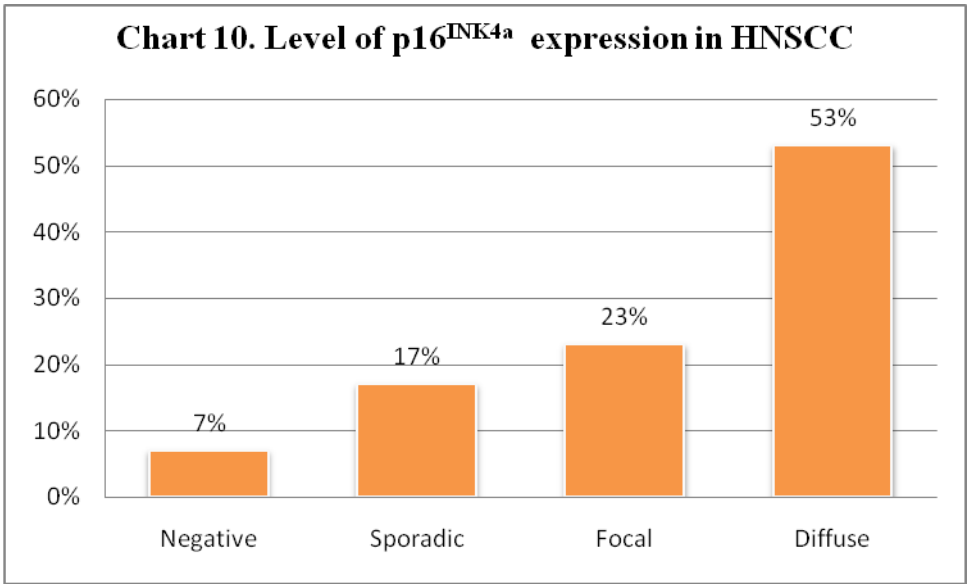
While observing the level of expression of $p16^{INK4a}$ by IHC, 93.33% cases of HNSCC were found to be positive (Table 9.). On observing the level of expression of $p16^{INK4a}$ in HNSCC, out of 60 cases 53.33% had diffuse pattern, followed by focal (23.33%) and sporadic (16.67%) (Table 13, Chart 10).

Further, out of the 32/60 cases having diffuse pattern of $p16^{INK4a}$ expression, 56.52% of grade 1, 51.43% of grade 2 and 50% of grade 3 HNSCC cases had diffuse pattern. Sporadic pattern of expression of $p16^{INK4a}$ was observed in 21.74%, 11.43% and 50% of cases among the HNSCC grade 1, grade 2 and grade 3 respectively (Table 13.).

Lesions	**Total n=60**	**Negative n=4**		**Sporadic n=10**		**Focal n=14**		**Diffuse n=32**		**P value**
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	
HNSCC Grade 1	23	1	4.35	5	21.74	4	17.39	13	56.52	0.667
HNSCC Grade 2	35	3	8.57	4	11.43	10	28.57	18	51.43	
HNSCC Grade 3	2	0	0	1	50	0	0	1	50	
Total	60	4	6.67	10	16.67	14	23.33	32	53.33	

Pearson Chi Square test

Table 13. Level of $p16^{INK4a}$ expression in different grades of HNSCC

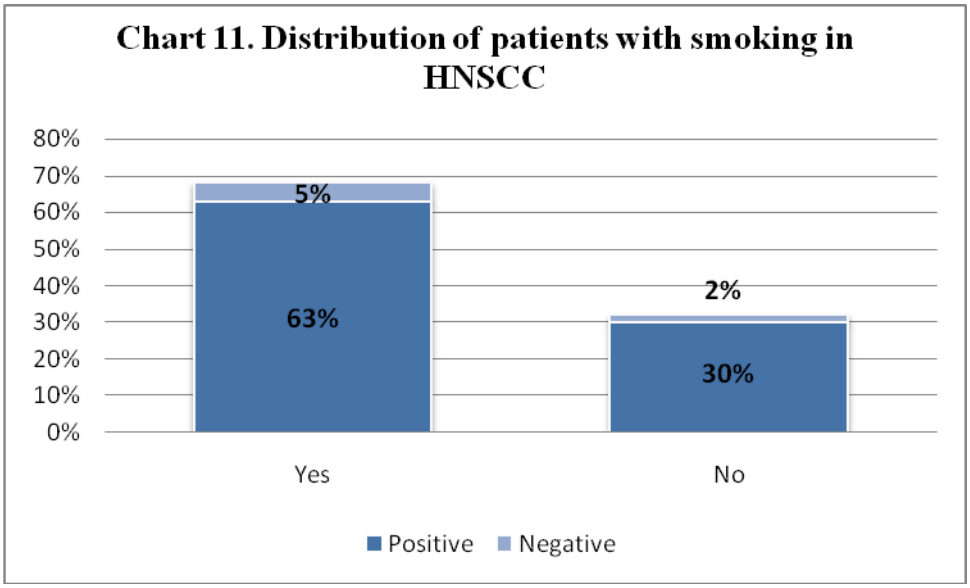


Among the 60 cases of HNSCC 44 cases (68%) had smoking history. Among the patients with smoking history 92.8% are $p16^{INK4a}$ positive (Table 14.).

Smoking	Total n=60	HNSCC				P value
		$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		
		n	%	n	%	
Yes	41	38	92.68	3	7.32	1.000
No	19	18	94.74	1	5.26	

Fisher's exact test

Table 14. Distribution of patients with smoking in HNSCC



Out of the 60 cases of HNSCC 7 cases (12%) had alcohol consumption history. Among the patients with alcohol consumption history 85.71% are $p16^{INK4a}$ positive (Table 15.).

Alcohol	Total n=60	HNSCC				P value
		$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		
		n	%	n	%	
Yes	7	6	85.71	1	14.29	0.399
No	53	50	94.34	3	5.66	

Fisher's exact test

Table 15. Distribution of patients with alcohol in HNSCC

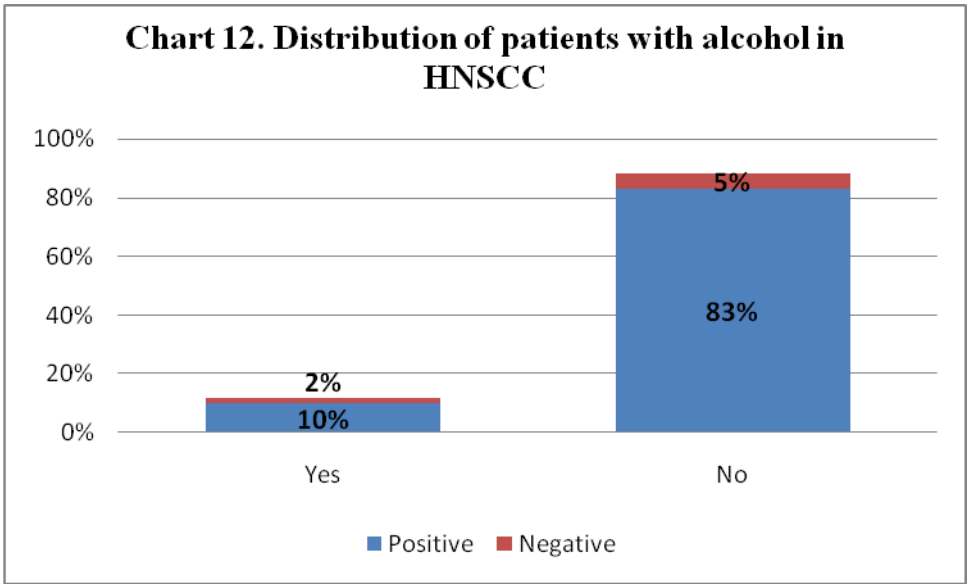


Chart 12. Distribution of patients with alcohol in HNSCC
100%
80%
60%
40%
20%
0%
2%
10%
5%
83%
Yes
No
Positive
Negative

COLOUR PLATES

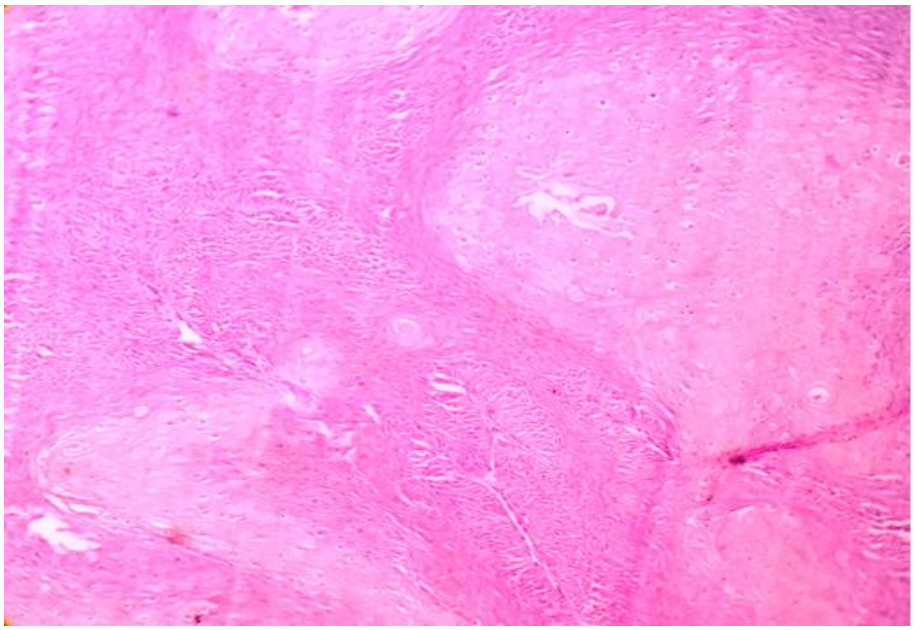


Fig. 1. WDSCC Well differentiated squamous cell carcinoma (WDSCC) – H&E staining (x100)

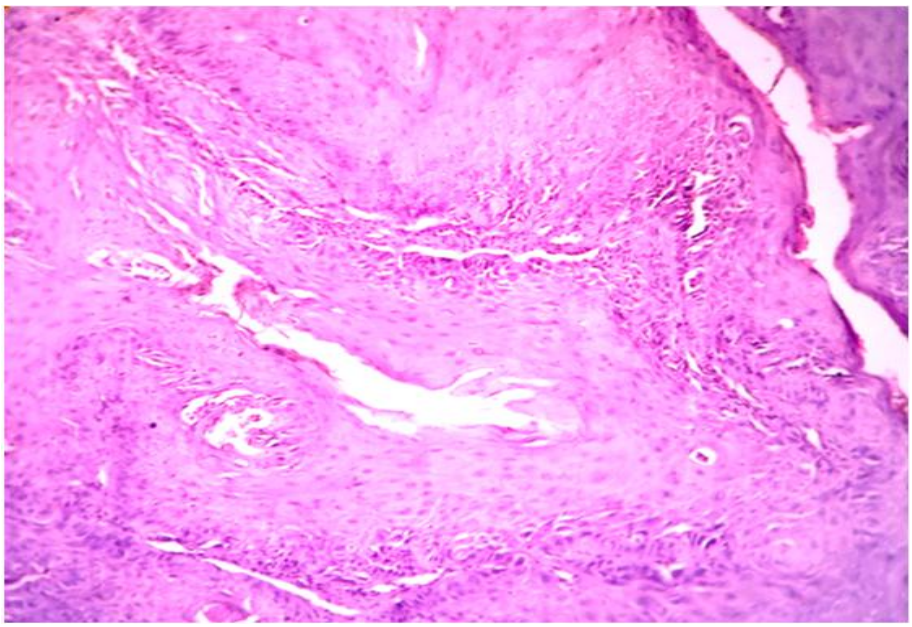


Fig. 2. Moderately differentiated squamous cell carcinoma (MDSCC) - H&E staining (x100)

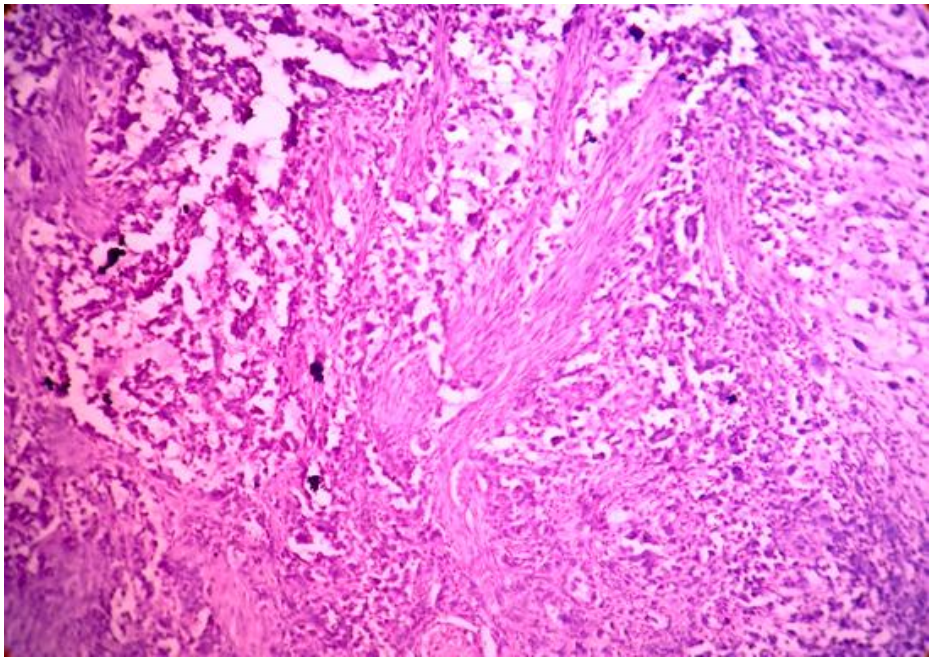


Fig. 3. Poorly differentiated squamous cell carcinoma (PDSCC)- H&E staining (x100)

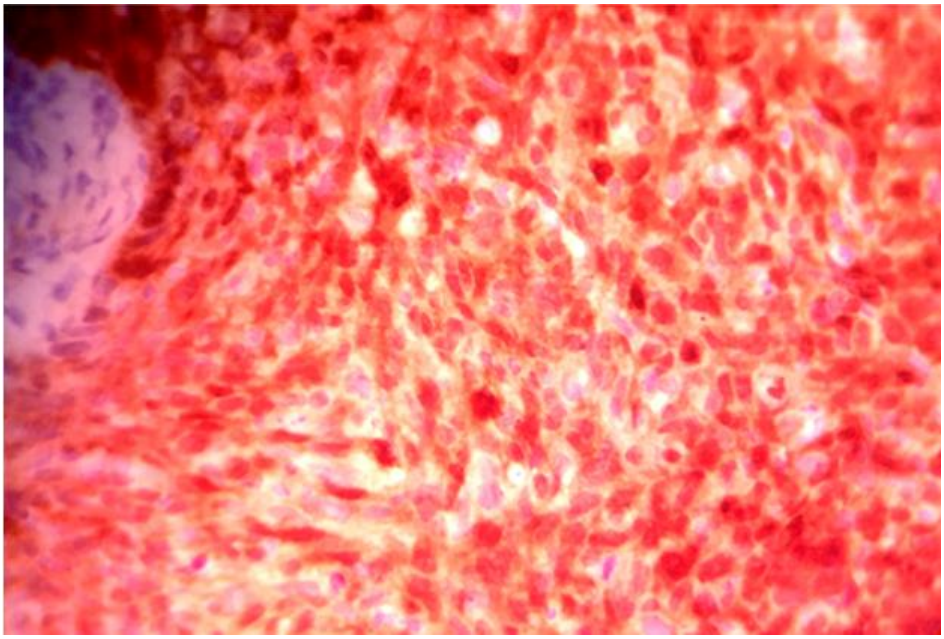


Fig. 4. WDSCC – Diffuse pattern of $p^{16INK4a}$ immunostaining (x400)

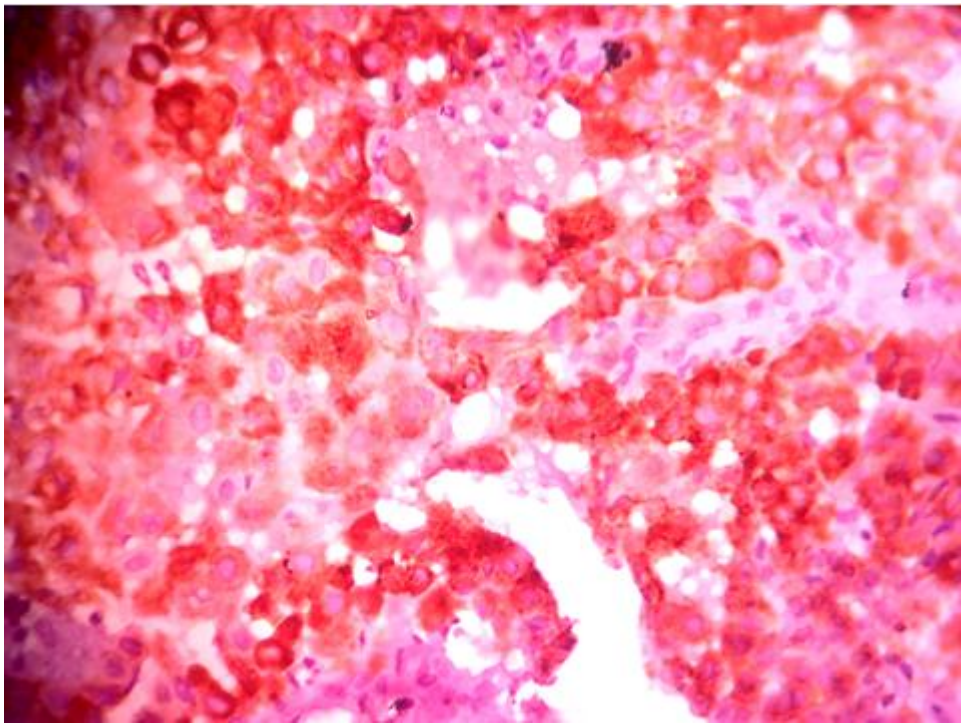


Fig. 5. MDSCC – Diffuse pattern of $p^{16INK4a}$ immunostaining (x400)

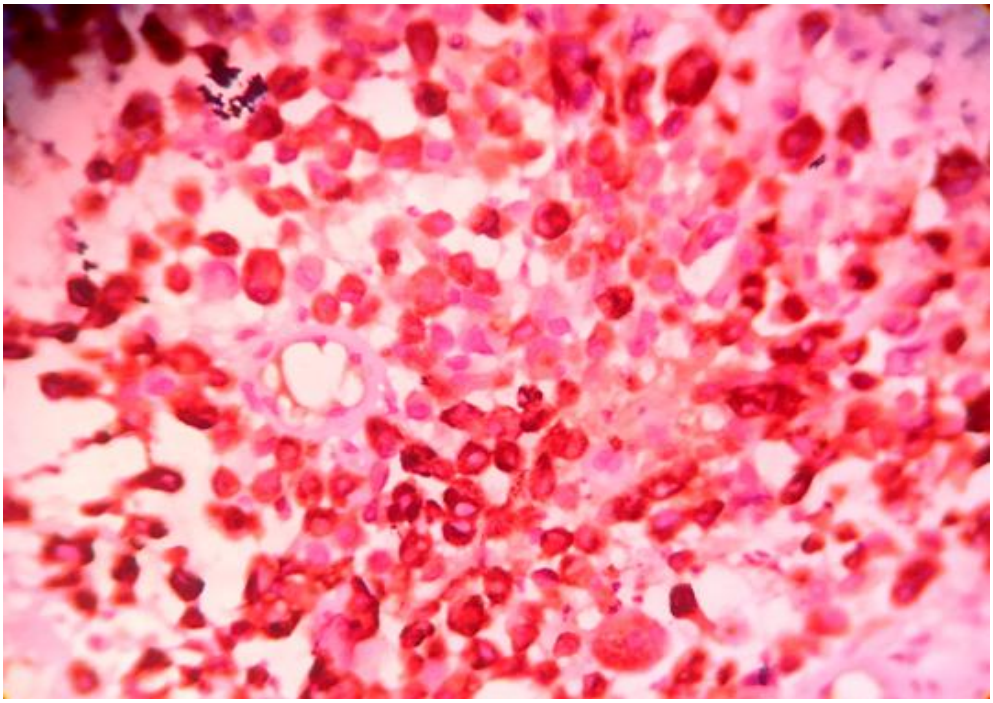


Fig. 6. PDSCC – Diffuse pattern of $p^{16INK4a}$ immunostaining (x400)

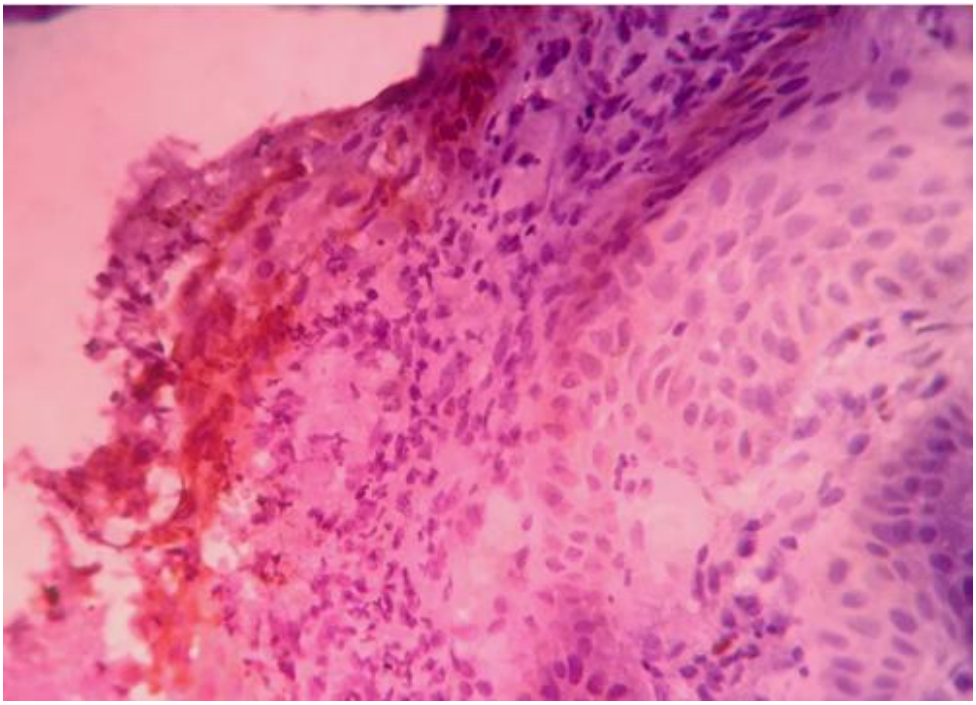


Fig. 7. WDSCC – Focal pattern of $p^{16INK4a}$ immunostaining (x400)

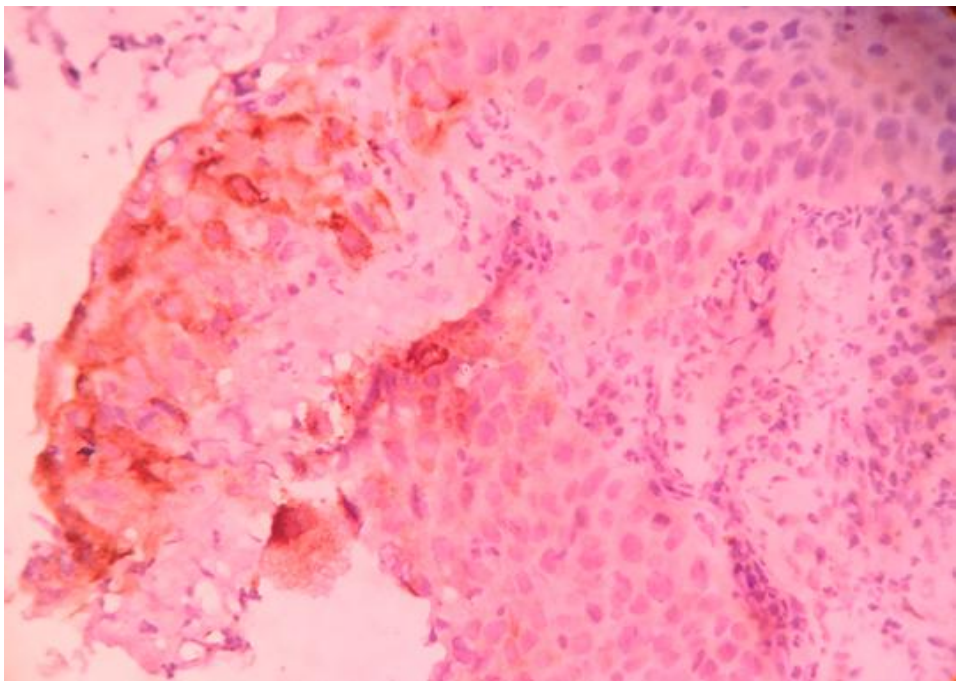


Fig. 8. MDSCC – Focal pattern of $p^{16INK4a}$ immunostaining (x400)

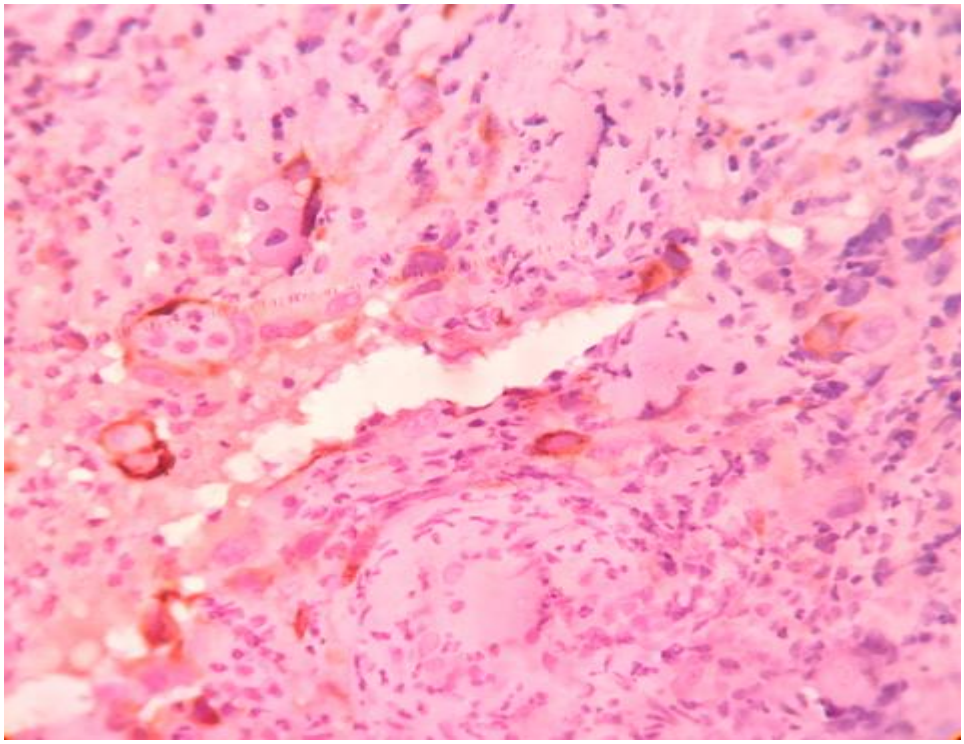


Fig. 9. WDSCC – Sporadic pattern of $p^{16INK4a}$ immunostaining (x400)

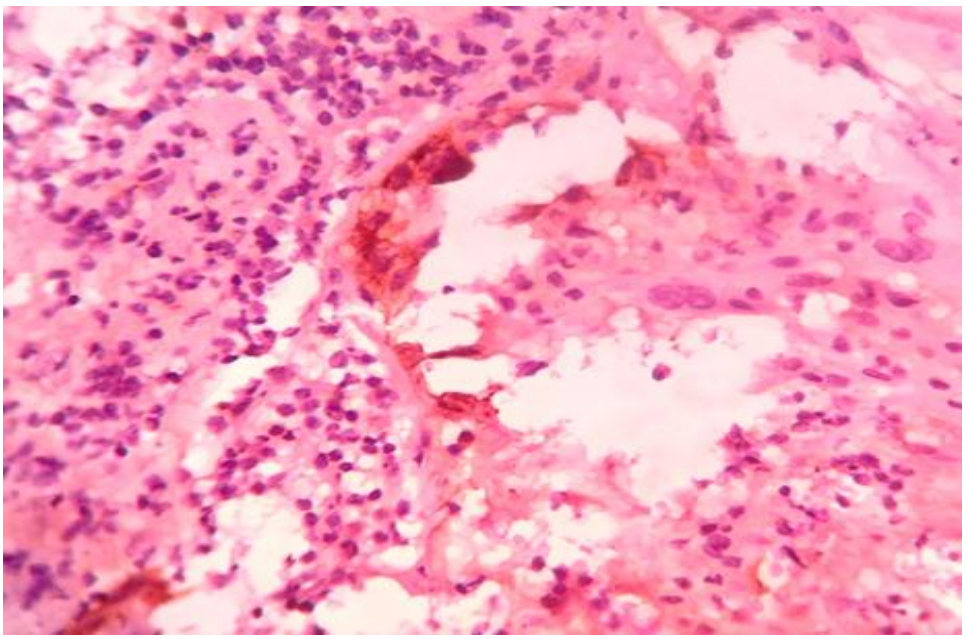


Fig. 10. MDSCC – Sporadic pattern of $p^{16INK4a}$ immunostaining (x400)

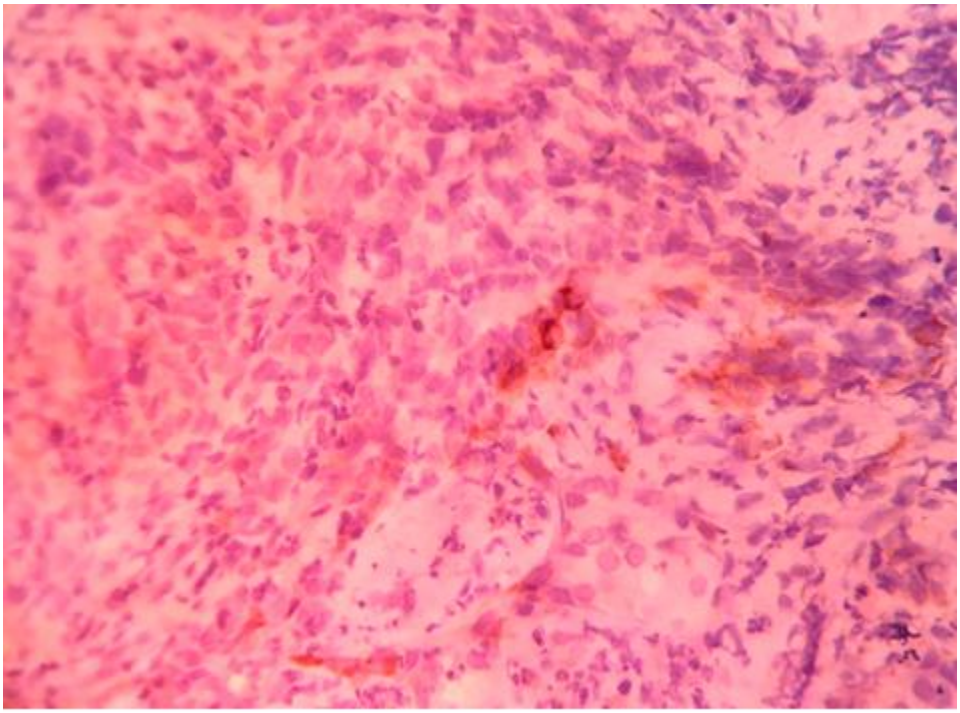


Fig. 11. PDSCC – Sporadic pattern of $p^{16INK4a}$ immunostaining (x400)

DISCUSSION

HNSCC continues to be a public health problem with an estimated incidence of 600 000 cases and 200 000 deaths annually[1].

In the present study, oropharynx was observed to be the commonest site affected by squamous cell carcinoma. Larynx was observed to be the least affected site.

The reports implicating specific HPV types in HNSCC were first published in 1985[126,127]. It has been observed that HPV 16 participates in disruption of regulation of $p16^{INK4a}$ suppressor protein and its over expression can be used as a surrogate marker for detection of HPV association in HNSCC. It has been observed that there are two groups of HNSCC, one group is associated with HPV infection ($p16^{INK4a}$ positive) and the other group is not associated with HPV infection ($p16^{INK4a}$ negative). In the HPV associated HNSCC two different mechanisms at the molecular level produce carcinogenesis via p53 and pRB[60]. The present study also indicates two groups of HNSCC on the basis of $p16^{INK4a}$ expression as 93.33% cases of HNSCC were positive for the over expression of $p16^{INK4a}$.

Further it has been observed that $p16^{INK4a}$ expression is a strong independent prognostic indicator also[114]. Patients with HNSCC not expressing $p16^{INK4a}$ had increased risk of death and increased risk of recurring cancer in comparison to those expressing it. Prognosis of $p16^{INK4a}$ positive cases has been reported to be better irrespective of histological grade.

Our study is a hospital based study and there is an increase in the percentage of $p16^{INK4a}$ positive HNSCC cases. According to Caihua Liang et al 2012, the prevalence of HNSCC based on PCR and $p16^{INK4a}$ detection based studies was 62%[128].

In the present study 75% of the cases are more than 50 years of age. According to Zeyi Deng et al 2014 86.67% cases are more than 50 years of age[129].

In the present study, among the 15 cases (25%) which are less than 50 years of age, 93.33% are $p16^{INK4a}$ positive. According to Zeyi Deng et al 2014 35% are $p16^{INK4a}$ positive[129] (Table 16.).

Our study	**Age group (years)**	**Total n=60**	**HNSCC**				**P value**
			$P16^{INK4a}$ +ve n=56		**$P16^{INK4a}$ -ve n=4**		
			n	**%**	**n**	**%**	
	Age≤50	15	14	93.33	1	6.67	1.000
	Age>50	45	42	93.33	3	6.67	
Zeyi Deng et al 2014	**Age group (years)**	**Total n=150**	**HNSCC**				**P value**
			$P16^{INK4a}$ +ve n=30		**$P16^{INK4a}$ -ve n=120**		
			n	**%**	**n**	**%**	
	Age≤50	20	7	35	13	65	0.128
	Age>50	130	23	17.7	107	82.3	

Table 16. Age distribution of patients with HNSCC - Comparison

The mean age for $p16^{INK4a}$ positive HNSCC cases in our study is 58.68 years. According to Zeyi Deng et al 2014 it is 61.8 years[129] and according to Caihua Liang et al 2012 it is56.4 years[128] (Table 17.).

The median age for $p16^{INK4a}$ positive and negative HNSCC cases in our study are 60 years and 58 years respectively. According to Gul Kanyilmaz et al 2015 it is 60 and 59 years respectively[130].

The Range of the age group for $p16^{INK4a}$ positive HNSCC cases in our study is 22 to 83 years. According to Gul Kanyilmaz et al 2015 it is 15 to 70 years[130]. According to Zeyi Deng et al 2014 it is 39 to 89 years[129] (Table 17.).

In our study, the beginning age in the $p16^{INK4a}$ positive HNSCC cases is 22 years. According to Gul Kanyilmaz et al 2015 it is 15 years[130]. According to Zeyi Deng et al 2014 it is 39 years[129].

Our study	**Age group (years)**	**HNSCC**		
		All patients n=60	**P16^{INK4a} +ve n=56**	**P16^{INK4a} -ve n=4**
	Range	22-83	22-83	44-75
	Mean±SD	58.68±12.83	58.68±12.94	58.75±12.91
	Median	60	60	58
Zeyi Deng et al 2014	**Age group (years)**	**HNSCC**		
		All patients n=150	**P16^{INK4a} +ve n=30**	**P16^{INK4a} -ve n=120**
	Range	28-89	39-89	28-86
	Mean	64.1	61.8	64.7
Gul Kanyilmaz et al 2015	**Age group (years)**	**HNSCC**		
		All patients n=131	**P16^{INK4a} +ve n=58**	**P16^{INK4a} -ve n=73**
	Range	15-82	15-70	17-82
	Median	60	60	59
Caihua Liang et al 2012	**Age group (years)**	**HNSCC**		
		P16^{INK4a} +ve n=54		**P16^{INK4a} -ve n=179**
	Mean±SD	56.4±9.2		60.3±12.3

Table 17. Age distribution of patients with HNSCC - Comparison

In our study, male cases among the total HNSCC cases is 85%. According to Gul Kanyilmaz et al 2015 it is 88.55%[130]. According to Zeyi Deng et al 2014 it is 84.67%[129]. According to Caihua Liang et al 2012 it is 71.24%[128] (Table 18.).

In our study, among the male patients 92.16% and among the female patients 100% are $p16^{INK4a}$ positive. According to Gul Kanyilmaz et al 2015 40.52% male patients and 73.33% female patients are $p16^{INK4a}$ positive[130]. According to Zeyi Deng et al 2014 18.9% male patients and 26.1% female patients are $p16^{INK4a}$ positive[129]. According to Caihua Liang et al 2012 27.7% male patients and 11.9% female patients are $p16^{INK4a}$ positive[128].

Our study	**Gender**	**Total n=60**	**HNSCC**				
			$P16^{INK4a}$ +ve n=56		**$P16^{INK4a}$ -ve n=4**		**P value**
			n	**%**	**n**	**%**	
	Male	51	47	92.16	4	7.84	1.000
	Female	9	9	100	0	0	
Gul Kanyilmaz et al 2015	**Gender**	**Total n=131**	**HNSCC**				
			$P16^{INK4a}$ +ve n=58		**$P16^{INK4a}$ -ve n=73**		**P value**
			n	**%**	**n**	**%**	
	Male	116	47	82	69	95	0.01
	Female	15	11	18	4	5	
Zeyi Deng et al 2014	**Gender**	**Total n=150**	**HNSCC**				
			$P16^{INK4a}$ +ve n=30		**$P16^{INK4a}$ -ve n=120**		**P value**
			n	**%**	**n**	**%**	
	Male	127	24	18.9	103	81.1	0.408
	Female	23	6	26.1	17	73.9	
Caihua Liang et al 2012	**Gender**	**Total n=233**	**HNSCC**				
			$P16^{INK4a}$ +ve n=54		**$P16^{INK4a}$ -ve n=179**		**P value**
			n	**%**	**n**	**%**	
	Male	166	46	27.7	120	72.3	0.01
	Female	67	8	11.9	59	88.1	

Table 18. Sex distribution of patients with HNSCC - Comparison

In our study, 5/9 female cases are ≤50 years and all are $p16^{INK4a}$ positive. 10/51 male cases are ≤50 years and 90% of which are $p16^{INK4a}$ positive. There is no much difference between $p16^{INK4a}$ positive cases in the ≤50 years and >50 years age groups (Table 8.).

In our study, the commonest site of the HNSCC oropharynx and the least common site is the nasopharynx. We did not receive biopsy from the nasopharynx during our study period. According to Zeyi Deng et al 2014 also the commonest site is the oropharynx and the least common site is the nasopharynx[129] (Table 19.).

In our study, the percentage of $p16^{INK4a}$ positive cases is highest (94.44%) in the oropharynx and lowest (91.67%) in the larynx. (No biopsy received from the nasopharynx). According to Zeyi Deng et al 2014, highest (37.7%) in the oropharynx and lowest (7.7%) in the hypopharynx[129].

	Site of the lesion	Total n=60	HNSCC				
			$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		P value
			n	%	n	%	
Our study	**Oral cavity**	17	16	94.12	1	5.88	0.988
	Oropharynx	18	17	94.44	1	5.56	
	Hypopharynx	13	12	92.30	1	7.70	
	Larynx	12	11	91.67	1	8.33	
	Total	60	56	93.33	4	6.67	
	Site of the lesion	**Total n=150**	**HNSCC**				
			$P16^{INK4a}$ +ve n=30		$P16^{INK4a}$ -ve n=120		P value
			n	%	n	%	
Zeyi Deng et al 2014	**Oral cavity**	24	2	8.3	22	91.7	0.002
	Nasopharynx	10	2	20	8	80	
	Oropharynx	53	20	37.7	33	62.3	
	Hypopharynx	39	3	7.7	36	92.3	
	Larynx	24	3	12.5	21	87.5	
	Total	150	30	20	120	80	

Table 19. Distribution of patients with HNSCC in different sites of Head and Neck region - Comparison

In our study, the percentage of $p16^{INK4a}$ positive cases in the oropharyngeal group is 94.44% (higher) and in the non-oropharyngeal group is 92.86% (lower). According to Zeyi Deng et al 2014, also higher (37.74%) in the oropharyngeal group and lower (10.31%) in the non-oropharyngeal group[129] (Table 20.).

Our study	**Site of the lesion**	**Total n=60**	**HNSCC**			
			P16^{INK4a} +ve n=56		**P16^{INK4a} -ve n=4**	
			n	**%**	**n**	**%**
	Oropharyngeal	18	17	94.44	1	5.56
	Non-oropharyngeal	42	39	92.86	3	7.14
Zeyi Deng et al 2014	**Site of the lesion**	**Total n=150**	**HNSCC**			
			P16^{INK4a} +ve n=30		**P16^{INK4a} -ve n=120**	
			n	**%**	**n**	**%**
	Oropharyngeal	53	20	37.74	33	62.26
	Non-oropharyngeal	97	10	10.31	87	89.69

Table 20. Distribution of patients with HNSCC in Oropharyngeal and Non-oropharyngeal region - Comparison

In our study, in the oral cavity squamous cell carcinoma, the most common site involved is tongue (64%) and the least common site involved is the alveolus and gingiva (each 6%). According to Pradyot Prakash et al 2013 the most common site is the tongue (37.7%) and the least common site is the hard palate and gingiva (each 1.45%)[131] (Table 21).

In our study, 90.91% of tongue squamous cell carcinomas were p16^{INK4a} positive (Table 11.).

Site of the lesion	Oral cavity SCC			
	Our study n=17		Pradyot Prakash et al 2013 n=69	
	n	%	n	%
Tongue	11	64	26	37.7
Buccal mucosa	2	12	17	24.6
Cheek	0	0	13	18.8
Alveolus	1	6	4	5.8
Lip	0	0	2	2.9
Angle of mouth	0	0	2	2.9
Soft palate	2	12	3	4.3
Hard palate	0	0	1	1.45
Gingiva	1	6	1	1.45

Table 21. Distribution of sites with SCC of Oral cavity - Comparison

In our study, $p16^{INK4a}$ positive cases are highest (100%) in the grade 3 HNSCC, followed by grade 1(95.65%) and lowest (91.43%) in the grade 2 HNSCC. According to Zeyi Deng et al 2014, highest (42.1%) in the grade 1 HNSCC, followed by grade 2(19%) and lowest (14.7%) in the grade 3 HNSCC[129] (Table 22.).

Our study	**Tumour differentiation**	**Total n=60**	**$P16^{INK4a}$ +ve n=56**		**$P16^{INK4a}$ -ve n=4**		**P value**
			n	**%**	**n**	**%**	
	HNSCC Grade 1	23	22	95.65	1	4.35	0.761
	HNSCC Grade 2	35	32	91.43	3	8.57	
	HNSCC Grade 3	2	2	100	0	0	
Zeyi Deng et al 2014	**Tumour differentiation**	**Total n=150**	**$P16^{INK4a}$ +ve n=30**		**$P16^{INK4a}$ -ve n=120**		**P value**
			n	**%**	**n**	**%**	
	HNSCC Grade 1	19	8	42.1	11	57.9	0.030
	HNSCC Grade 2	63	12	19	51	81	
	HNSCC Grade 3	68	10	14.7	58	85.3	

Table 22. $p16^{INK4a}$ expression in different grades of HNSCC - Comparison

In our study, among the HNSCC cases, most (53.33%) are having diffuse pattern of $p16^{INK4a}$ expression, followed by focal (23.33%) and lowest (16.67%) having sporadic pattern of expression. In our study, in the oral cavity squamous cell carcinoma, most (35.30%) are having diffuse pattern, followed by focal and sporadic patterns, each having 29.41%. According to Pradyot Prakash et al 2013, in the oral cavity squamous cell carcinoma, diffuse pattern is the most common (31.9%), followed by sporadic (24.6%) and lowest (14.5%) having focal pattern of expression[131] (Table 23.).

Our study	**Site of the lesion**	**Oral cavity SCC**							
		Negative		**Sporadic**		**Focal**		**Diffuse**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
	Total n=17	1	5.88	5	29.41	5	29.41	6	35.30
Pradyot Prakash et al 2013	**Site of the lesion**	**Oral cavity SCC**							
		Negative		**Sporadic**		**Focal**		**Diffuse**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
	Total n=69	20	29	17	24.6	10	14.5	22	31.9

Table 23. Level of $p16^{INK4a}$ expression in Oral cavity SCC - Comparison

In our study, among the $p16^{INK4a}$ positive HNSCC cases 92.68% were having smoking habit. According to Gul Kanyilmaz et al 2015 45.92% of $p16^{INK4a}$ positive cases were having smoking habit[130]. According to Caihua Liang et al 2012 19.2% were having smoking habit[128] (Table 24.).

Our study	**Smoking**	**Total n=60**	**HNSCC**				
			P16^{INK4a} +ve n=56		**P16^{INK4a} -ve n=4**		**P value**
			n	**%**	**n**	**%**	
	Yes	41	38	92.68	3	7.32	1.000
	No	19	18	94.74	1	5.26	
Gul Kanyilmaz et al 2015	**Smoking**	**Total n=131**	**HNSCC**				
			P16^{INK4a} +ve n=58		**P16^{INK4a} -ve n=73**		**P value**
			n	**%**	**n**	**%**	
	Yes	98	45	77	53	72	ns
	No	33	13	23	20	28	
Caihua Liang et al 2012	**Smoking**	**Total n=233**	**HNSCC**				
			P16^{INK4a} +ve n=54		**P16^{INK4a} -ve n=179**		**P value**
			n	**%**	**n**	**%**	
	Yes	187	36	19.2	151	80.8	<0.01
	No	46	18	39.1	28	60.9	

Table 24. Distribution of patients with smoking in HNSCC – Comparison

ns – not significant

In our study, among the p16^{INK4a} positive HNSCC cases 85.71% were having alcohol consuming habit. According to Gul Kanyilmaz et al 2015 35.71% of p16^{INK4a} positive cases were having alcohol consuming habit[130] (Table 25.).

Our study	Alcohol	Total n=60	HNSCC				P value
			$P16^{INK4a}$ +ve n=56		$P16^{INK4a}$ -ve n=4		
			n	%	n	%	
	Yes	7	6	85.71	1	14.29	0.399
	No	53	50	94.34	3	5.66	
Gul Kanyilmaz et al 2015	**Alcohol**	**Total n=131**	**HNSCC**				
			$P16^{INK4a}$ +ve n=58		$P16^{INK4a}$ -ve n=73		P value
			n	%	n	%	
	Yes	14	5	9	9	12	ns
	No	117	53	91	64	88	

Table 25. Distribution of patients with alcohol in HNSCC - Comparison

ns – not significant

SUMMARY

The incidence of HPV associated HNSCC is increasing over the past 30 years. It is a growing public health concern. It has been reported that tissues of HPV associated HNSCCs over express $p16^{INK4a}$. Therefore $p16^{INK4a}$ is used as a surrogate marker to detect HPV associated HNSCC. Immunohistochemical detection of $p16^{INK4a}$ is an easy and simple technique than molecular detection of HPVs. Hence we investigated the presence of $p16^{INK4a}$ in HNSCCs.

The objectives of our study are (1)To study the association of $p16^{INK4a}$ expression with HNSCC, thus with the HPV. (2)To compare the $p16^{INK4a}$ expression in different sites of the HNSCC. (3)To correlate the level of $p16^{INK4a}$ expression with different grades of HNSCC.

A total sample of 60 cases were analysed during the period of June 2014 to August 2015. We performed IHC detection in sections of formalin fixed paraffin embedded tissue of HNSCC cases and correlated the various patterns of $p16^{INK4a}$ positivity with respect to histopathological diagnosis.

In the present study, increased number of HNSCC cases were seen overexpressing $p16^{INK4a}$ (93.33%). Oropharynx was the most common site for $p16^{INK4a}$ positivity in HNSCC cases (94.44%). Among the oral cavity SCC cases, tongue was the most common site involved (64%). Of the HNSCC cases, most cases (53.33%) had diffuse pattern of $p16^{INK4a}$ overexpression. However, DNA detection based studies are needed to validate the utility of IHC detection of $p16^{INK4a}$ as a surrogate marker for HPV associated HNSCC.

CONCLUSION

The present cross-sectional study "Detection of $p16^{INK4a}$ in Oropharyngeal and Upper Respiratory Tract Squamous Cell Carcinoma" was conducted in the Department of pathology from June 2014 to August 2015.

The present study demonstrated increased association of $p16^{INK4a}$ over expression in cases of HNSCC (93.33%). HNSCC was more common in males with male to female ratio of 6 : 1 . Oropharynx accounted for the most common site of occurrence of HNSCC(30%). Also oropharynx was the most common site for $p16^{INK4a}$ positivity in HNSCC cases (94.44%).

Among the oral cavity SCC cases, tongue was the most common site involved (64%). Among the $p16^{INK4a}$ positive cases most cases are HNSCC Grade 2 (53%). Of the HNSCC cases, most cases (53.33%) had diffuse pattern of $p16^{INK4a}$ over expression. Diffuse pattern of $p16^{INK4a}$ over expression was most common in HNSCC Grade 1 cases (56.52%).

Further, DNA detection based studies are needed to validate the utility of IHC detection of $p16^{INK4a}$ as a surrogate marker for HPV associated HNSCC.

In future, prophylactic vaccination for boys and girls before the starting of sexual activity will prevent HPV infection and thus reduce the incidence of HPV associated HNSCC. Plans to improve public awareness and knowledge of clinical features and risk factors will reduce the disease burden of HPV associated HNSCC.

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ANNEXURE - I

PROFORMA

Date:

1. Name : OP/IP No :

2. Age :

3. Sex : Male Female

4. History : Smoking – Yes / No Alcohol – Yes / No

Clinical Diagnosis :

Site of the lesion :

Histopathological confirmation and grading of H&E stained section

HNSCC Grade 1

HNSCC Grade 2

HNSCC Grade 3

$p16^{INK4a}$ expression

Negative

Positive

Sporadic

Focal

Diffuse

MASTER CHART

S. No.	Path No.	Age	Age group	Sex	Site - HNSCC	Site -oral SCC	Clinical diagnosis	HPE diag	IHC diag	Smoking	Alcohol
1	G220/14	57	2	1	4	0	Growth Supraglottis	2	3	1	2
2	G227/14	57	2	1	1	5	Growth Gingiva	2	2	1	2
3	G237/14	70	2	1	2	0	Growth Rt Tonsil	2	1	1	1
4	G268/14	55	2	1	1	1	Growth Tongue	1	2	1	2
5	G295/14	80	2	1	3	0	Growth Hypopharynx	1	0	1	2
6	G310/14	49	1	1	2	0	Growth Oropharynx	1	3	1	2
7	G329/14	52	2	1	2	0	Growth Oropharynx	2	0	1	2
8	G389/14	52	2	1	3	0	Growth Lt Pyriform fossa	2	3	1	2
9	G431/14	50	1	1	1	2	Growth Lt Buccal mucosa	1	1	1	2
10	G527/14	52	2	1	1	1	Growth Tongue	1	1	1	1
11	G528/14	54	2	1	2	0	Growth Oropharynx	1	1	2	2
12	G529/14	80	2	1	4	0	Growth Supraglottis	2	1	2	2
13	G652/14	45	1	1	1	1	Growth Tongue	2	2	1	2
14	G665/14	63	2	1	3	0	Rt Pyriform fossa	1	3	2	2
15	G788/14	50	1	1	2	0	Growth Rt Tonsil	1	2	1	2
16	G802/14	67	2	1	1	1	Growth Tongue	2	3	2	2
17	G920/14	67	2	1	1	1	Growth Tongue	1	3	1	2
18	G922/14	75	2	1	2	0	Growth Tonsillar fossa	2	2	1	2
19	G1000/14	60	2	1	2	0	Growth Oropharynx	1	1	2	2
20	G1043/14	65	2	1	4	0	Transglottic growth	1	3	1	2

S. No.	Path No.	Age	Age group	Sex	Site - HNSCC	Site -oral SCC	Clinical diagnosis	HPE diag	IHC diag	Smoking	Alcohol
21	G1046/14	60	2	1	1	1	?Ca Tongue	2	2	1	2
22	G1221/14	50	1	1	2	0	Growth Oropharynx	2	2	1	2
23	G1286/14	55	2	1	2	0	Growth Lt Tonsil	1	3	1	2
24	G1310/14	77	2	1	2	0	Growth Oropharynx	2	3	2	1
25	G1538/14	22	1	2	4	0	Growth Vocal cord	2	2	2	2
26	G1550/14	60	2	1	4	0	Growth Vocal cord	2	0	2	1
27	G1587/14	65	2	2	3	0	Growth Rt Pyriform fossa	1	3	2	2
28	G1597/14	65	2	1	3	0	Growth Lt Pyriform fossa	1	3	1	2
29	G1736/14	75	2	1	4	0	Growth Supraglottis	2	2	1	2
30	G1755/14	60	2	1	2	0	Growth Oropharynx	2	2	1	2
31	G1763/14	23	1	2	2	0	Growth Oropharynx	2	3	2	2
32	G1766/14	50	1	1	4	0	Growth Supraglottis	2	3	1	2
33	G8/15	50	1	1	1	3	Growth Alveolus	2	1	1	2
34	G47/15	44	1	1	1	1	?Ca Tongue	2	0	1	2
35	G68/15	63	2	1	3	0	Growth Hypopharynx	2	2	1	2
36	G136/15	55	2	1	2	0	Growth Oropharynx	1	2	1	2
37	G138/15	37	1	1	3	0	Growth Hypopharynx	1	3	2	2
38	G187/15	58	2	1	3	0	? Ca Hypopharynx	2	3	1	1
39	G262/15	70	2	1	1	1	Growth Tongue	3	3	1	2
40	G301/15	69	2	1	2	0	Growth Oropharynx	2	2	1	2

S. No.	Path No.	Age	Age group	Sex	Site - HNSCC	Site -oral SCC	Clinical diagnosis	HPE diag	IHC diag	Smoking	Alcohol
41	G355/15	56	2	1	3	0	Growth Hypopharynx	2	3	1	2
42	G470/15	65	2	1	3	0	Growth Hypopharynx	1	3	1	2
43	G501/15	35	1	2	1	1	Ulcer Tongue	1	1	2	2
44	G551/15	60	2	1	2	0	Growth Oropharynx	3	1	1	2
45	G552/15	50	1	1	3	0	Growth Hypopharynx	2	3	1	2
46	G588/15	40	1	2	1	2	?Ca Rt Buccal vestibular	1	3	2	2
47	G594/15	55	2	1	1	4	Ulcer Soft plalate	2	3	1	2
48	G670/15	58	2	2	3	0	Growth Hypopharynx	2	3	2	2
49	G676/15	79	2	2	1	4	Growth Solft palate	1	2	2	2
50	G721/15	83	2	1	1	1	Ca Tongue	2	1	1	2
51	G743/15	61	2	2	4	0	Growth Epiglottis	2	3	2	2
52	G790/15	61	2	1	4	0	Growth Supraglottis	2	3	1	2
53	G803/15	60	2	1	2	0	Growth Oropharynx	2	3	2	2
54	G824/15	75	2	1	2	0	Growth Oropharynx	2	3	1	1
55	G892/15	65	2	1	1	1	? Ca Tongue	1	3	2	2
56	G964/15	70	2	1	4	0	Growth Lt Vocal cord	1	3	1	1
57	G1058/15	40	1	2	3	0	Growth Hypopharynx	2	3	2	2
58	G1081/15	60	2	1	4	0	Growth Epiglottis	1	3	1	2
59	G1107/15	70	2	1	4	0	Growth Supraglottis	2	3	1	2
60	G1117/15	70	2	1	2	0	Growth Oropharynx	2	3	1	2

ABBREVIATIONS FOR MASTER CHART

Age group

1. Age≤50 years
2. Age>50 years

Sex

1. Male
2. Female

Site – HNSCC – Head and Neck Squamous Cell Carcinoma Site

1. Oral cavity
2. Oropharynx
3. Hypopharynx
4. Larynx

Site – Oral SCC – Oral cavity Squamous cell Carcinoma

0. Not part of the oral cavity
1. Tongue
2. Buccal mucosa
3. Alveolus
4. Soft palate
5. Gingiva

HPE diag – Histopathological diagnosis

1. HNSCC Grade 1
2. HNSCC Grade 2
3. HNSCC Grade 3

IHC diag - $P^{16INK4a}$ expression

0. Negative
1. Sporadic
2. Focal
3. Diffuse

Smoking – Smoking habit

1. Yes
2. No

Alcohol –Alcohol consuming habit

1. Yes
2. No

ANNEXURE – III

GLOSSARY

CDKN2A	:	Cyclin dependent kinase inhibitor 2A
DAB	:	Di amino benzidine
EGFR	:	Epidermal growth factor receptor
FFPE	:	Formalin fixed paraffin embedded
HNSCC	:	Head and neck squamous cell carcinoma or Oropharyngeal and upper respiratory tract squamous cell carcinoma
HPV	:	Human papilloma virus
H&E	:	Haematoxylin & Eosin
IHC	:	Immunohistochemistry
ISH	:	In Situ hybridisation
MDSCC	:	Moderately differentiated squamous cell carcinoma
OPSCC	:	Oropharyngeal squamous cell carcinoma
PCR	:	Polymerase chain reaction
PBS	:	Phosphate buffer solution
PDSCC	:	Poorly differentiated squamous cell carcinoma
RT-qPCR	:	Real time quantitative polymerase chain reaction
SCC	:	Squamous cell carcinoma
TBS	:	Tris buffer solution
WDSCC	:	Well differentiated squamous cell carcinoma